GLYCEROL-FREEZING TECHNIQUE AS AN AID IN BLOOD TYPING OF WHALES*

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This paper describes the application of the glycerol-freezing preservation technique to blood type studies on whales. Following the lead of recent trends in anthropology (cf. Mourant, 1954), blood type antigens are receiving increasing attention as "genetic tags" useful as aids in the definition of intraspecific populations of marine animals (cf. Fujino, 1956; Ridgway et al., 1958; Suzuki et al., 1958). Method for preserving intact erythrocytes between the time of their collection and serological characterization are therefore of considerable interest. In contrast to samples of human or animal bloods which often can be taken and preserved under controlled conditions (Stormont et al., 1958; Strumia, 1958), the erythrocytes of marine forms are usually collected where sterile and other precautions are not practical. (Of note is the fact that whale erythrocytes can be preserved for one month or longer by the addition of the antimicrobial, guanofuracin, 5-Nitro-2-furfurylidine-amino-guanidine Hydrochloride, Toyama Chemical Co., Tokyo).

Preliminary studies on fish (Cushing, 1956; Cushing et al., 1957) having shown that the glycerol-freezing technique offered considerable promise, efforts were made to learn of its suitability for large scale research. This technique has been in use in various laboratories concerned with human and domestic animal blood type studies since it was first applied to blood bank research (cf. Kabat, 1956, p. 92 for a review of this subject). The general method employed here is based on consideration of the various researches noted above. The typing techniques and methods used are the same as described in earlier papers on the blood typing of marine animals (see above). (Of basic significance are the facts that erythrocytes hemolyze completely if frozen and thawed in the absence of glycerol (or related preservatives such as ethylene glycol), and that glycerol inhibits agglutination reactions very markedly in concentrations suitable for preservation.

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The application of the glycerol-freezing technique will be taken up by separate species.

Finback whales (*Balaenoptera physalus*).

Samples of blood from finback whales were first preserved by glycerol-freezing in the summer of 1957, when six were obtained through the courtesy of Mr. Charles Caito, Whaling Station, Del Monte Fishing Co., San Francisco, California. There were part of a collection made by Mr. Caito from whales captured off San Francisco by his company. The success of these efforts and of a small collection of finback samples taken in 1957 (Fujino) led to the collection of 460 finback samples from the north Pacific during the months of June, July and August 1958. This collection was made under the supervision of Mr. K. Nasu, Whales Research Institute, Tokyo.

Samples from these whales were taken during the flensing of carcasses on the factory ship. While these whales had been dead for from a half to twenty six hours (seven and a half hours in average) at the time of sampling, the biological log showed that this variability had no effect on subsequent sample condition. 2.5 ml. or 125 ml. of whole blood were mixed with an approximately equal volume of a solution consisting of four parts of glycerol and six parts of a five percent solution of trisodium citrate. (As an additional precaution against accidental warming guanofuracin had been added to this mixture at 200 mg. per 100 ml. However, the use of antimicrobials is not essential to the success of the glycerol-freezing method). The samples were kept at 5°C for 2 to 16 days on the factory ship until a box of 50 was accumulated. This box was then transferred to a refrigerator ship and kept at −20°C until the eventual recovery of samples for study at Tokyo University.

Analysis started four months after collection of the last samples and included a set of fifteen 125 ml. samples taken eight months before in the Antarctic. Intact erythrocytes were recovered by dialysis at room temperatures for two hours against 1.5% saline, followed by several times' washing. Cells prepared in this manner remained in good condition for several days at refrigeration temperatures, being rewashed at the start of each day's work. It was found convenient to handle from fifteen to twenty 10 ml. aliquots of samples per day, but this number could have been greatly expanded with additional assistance. Aliquots could be removed with a spatula without thawing samples, or obtained by thawing the samples under running tap water. Several freezings and thawings did not appear to affect the fragility of the cells.

Varying degrees of initial hemolysis were found in almost all samples, but good yields of cells were obtained from all the large samples

(total=15 bottles), and from all but 30 of the small samples (total=450 bottles). Of these 19 came from a single box of fifty which apparently had not been refrigerated properly. The reasons for hemolysis have not yet been studies, but it was found that smaller samples (e.g. 5 ml.) showed more tendency to hemolyze than larger ones (e.g. 250 ml). In addition to hemolysis, the yield of cells also is influenced by internal sedimentation and clotting in the body of the whale following death, so that care should be taken to collect from blood vessels that appear to contain relatively high concentrations of erythrocytes.

The Ju system of antigens (Fujino, 1956, 1958) was selected for detailed study. The authors have found no reason to believe that either the specificities or titers of the serological reactions of this system were altered during the period of freezing. Various absorptions also showed that the cells were as useful in this work as unfrozen ones. Table 1 shows that the frequencies of antigens as distributed

TABLE 1. COMPARISON OF FREQUENCIES OF FINBACK Ju TYPES EXAMINED BY FRESH AND GLYCEROL PRESERVED CELLS

Area	East China Sea				off Kamchatka				off Navarin			
Ju blood type	1	1.2	2	Sum	1	1.2	2	Sum	1	1.2	2	Sum
Fresh cells a)	95	13	21	129 c)	329	27	73	429 d)	—	—	—	no data
Frozen cells b)	—	—	—	no data	113	1	5	119 e)	71	2	3	76

Area	off Oleutorskie				South of east Aleutian				North of east Aleutian			
Ju blood type	1	1.2	2	Sum	1	1.2	2	Sum	1	1.2	2	Sum
Fresh cells a)	—	—	—	no data	29	1	2	32	1401	9	17	1427
Frozen cells b)	13	0	0	13	167	4	6	177	74	0	1	75 f)

a) East China Sea examined in 1956 and 1957, other areas in 1954 and 1955.
b) Examined in 1958 only.
c) Fujino, unpublished data.
d) Yearly and seasonal fluctuations have been observed.
e) May be distinguished into two separate operating seasons in 1958 in which seasonal fluctuations were seen.
f) May be distinguished into two operating seasons in 1958 in which no seasonal fluctuation was seen.

in the north Pacific finbacks are comparable with those observed in earlier studies.

Various observations suggest that cells with Ju 2 specificities may tend to slightly more fragile than those of Ju 1 type. These include field observations on fresh cells, and laboratory observations on recovered cells. This possibility has not yet been confirmed, but it is important to keep in mind in general, as the rejection of badly hemolyzed samples without typing might conceivable bias frequency counts in future studies. In this particular study, observations on the agglutination of ghosts and residual cells, as well as inhibition tests (Table 2), made it possible to type all the badly hemolysed samples.

TABLE 2. EXAMPLES OF THE AGGLUTINATION-INHIBITION TESTS OF FINBACK Ju 2 TYPE CELLS (NO. 14) BY SUCCESSIVE DILUTIONS OF THE DIALYSED SUPERNATANT FROM Ju 2 TYPE WHOLE BLOODS (NO. 14)

Dilutions of no. 14 supernatant:	Dilution of horse serum				
	32	64	128	256	512
2	+	−	−	−	−
4	+	−	−	−	−
8	+	+	−	−	−
16	++	+	±	−	−
32	+++	+++	+	−	−
64	+++	+++	++	+	−
Saline control	+++	+++	++	++	+

Each test was made by mixing one drop of supernatant with one drop of serum diluted, and fifteen minutes later, adding one drop of cell suspension. Reaction were read thirty minutes later.

The availability of frozen cells also made it possible to discover antibodies in the normal serums of the horse, pig and sheep (only one individual each was studied) that reacted specifically with cells carrying the Ju 2 factor. The use of horse and pig serum, combined with rabbit immune serum, has revealed variations in the agglutination titer of Ju 2 cells of different types that resemble similar variations in other species. Examples of these differences are shown in Table 3. At present it is not known whether these are due to quantitative or qualitative variations among the factors involved. The specificity and relative avidity of horse and pig serums for different Ju types were found to parallel exactly the reactions of immune serums with these types. All these serums were used in this series of typings and the relative frequencies of homozygotes and heterozygotes were found to be same as in previous years.

Inspection of these observed relative frequencies (Table 1) shows that they deviate from those that would be expected for a two allele system

TABLE 3. VARIATIONS IN THE AGGLUTINATION TITERS OF CELLS WITH DIFFERENT FINBACK Ju SPECIFICITIES

a) Examples of reactions with normal animal serums

Finback whale cells:		Dilution of animal serums: Horse						Pig						
		16	32	64	128	256	512	4	8	16	32	64	128	256
Ju 2 type	No. 2	###	###	###	###	+	+	###	###	###	###	###	##	+
	No. 14	###	###	###	###	##	+	###	###	###	###	###	##	+
Ju 1 Ju 2 type	No. 116	###	##	+	−	−	−	##	+	−	−	−	−	−
	No. 184	+	+	−	−	−	−	##	±	−	−	−	−	−
Ju 1 type	No. 12	−	−	−	−	−	−	−	−	−	−	−	−	−
	No. 13	−	−	−	−	−	−	−	−	−	−	−	−	−

b) Examples of reactions with immune serums

Finback whale cells:		Dilution of anti-Ju 2 immune no. 34 serum: Unabsorbed								Absorbed by Ju 1 cells				
		80	160	320	640	1280	2560	5120	10240	80	160	320	640	1280
Ju 2 type	No. 2	###	###	###	###	###	##	##	+	###	###	##	##	+
	No. 14	###	###	###	###	###	###	##	+	###	###	###	##	+
Ju 1 Ju 2 type	No. 116	###	###	###	##	+	−	−	−	###	+	−	−	−
	No. 184	###	###	##	+	+	−	−	−	###	±	−	−	−
Ju 1 type	No. 12	##	+	±	−	−	−	−	−	−	−	−	−	−
	No. 13	##	+	−	−	−	−	−	−	−	−	−	−	−

Dilution of anti-Ju 1 immune no. 47 serum

Finback whale cells:		Unabsorbed					Absorbed by Ju 2 cells			
		80	160	320	640	1280	5	10	20	40
Ju 2 type	No. 2	###	###	###	##	+	−	−	−	−
	No. 14	###	###	###	##	+	−	−	−	−
Ju 1 Ju 2 type	No. 116	###	###	###	##	+	###	##	+	−
	No. 184	###	###	###	##	+	###	##	+	−
Ju 1 type	No. 12	###	###	###	##	+	###	##	+	−
	No. 13	###	###	###	##	+	###	##	+	−

of antigens in populations in Hardy-Weinberg equilibria. It is too early to state which of the several possible factors that might be expected to cause this deviation are actually the responsible ones. (cf. Srb and Owen, 1952 for an introductory discussion of the Hardy-Weinberg equilibrium).

Blue white dolphins (Stenella caeruleo-albus).

Blood samples were taken from thirty blue white dolphins captured January 1, 1959 on the east side of the Izu Peninsula, Shizuoka Pref., Japan. These samples, 25 ml. of whole blood each, were collected into glycerol solution, frozen with dry ice and returned to Tokyo. A parallel set of samples from the same individuals was collected into citrate-guanofuracin solution, as described above, and returned to Tokyo unfrozen. Almost all samples gave good yields of cells after one month,

and the frozen samples gave good yields of cells after three months. The agglutinin titers and specificities of cells obtained at this time did not differ materially from those of freshly collected, unfrozen and frozen cells as determined in January. These dolphin cells were used in various studies, including the work on radioactive antibodies reported in this volume (Fujino et al., 1959).

Humpback whale (Megaptera nodosa).

Cells from twenty five humpback whales were among the samples obtained from Mr. Charles Caito at San Francisco. These were preserved by freezing (50 ml. of whole blood per sample) and almost all gave sufficient cells for study, although like the finback whales, varying degrees of hemolysis occurred.

A second collection of one hundred and five humpback whale bloods (25 ml. of whole blood per sample) were obtained from shore stations on Okinawa, Ryukyu Islands. These were collected under the supervision of Dr. M. Nishiwaki, Whales Research Institute, whose special efforts in this regard are greatly appreciated (see Nishiwaki, this volume). These samples were stored at −20°C and were returned to Tokyo by air where they were examined two months after collection. Sufficient cells were obtained from all samples excepting 5. Of these, 3 were left in the room temperatures (20∼22°C) before freezing for 8 hours or longer in which decomposition had started.

Table 4 shows the reactions of anti-finback Ju 2 no. 34 serum (sent to California by Fujino) with finback and humpback erythrocytes collected

TABLE 4. THE REACTIONS OF ANTI-FINBACK Ju 2 No. 34 SERUM WITH FINBACK AND HUMPBACK ERYTHROCYTES COLLECTED OFF SAN FRANCISCO, U.S.A.

	Unabsorbed				Absorbed by finback Ju 1 cells 4:			
Finback cells:	50	100	200	400	50	100	200	400
Ju 1 (4)	ⅲ	ⅲ	ⅲ	+	−	−	−	−
Ju 1 (10)	ⅲ	ⅲ	ⅱ	+	−	−	−	−
Ju 1 (25)	ⅲ	ⅱ	+	−	−	−	−	−
Ju 2 (31)	ⅲ	ⅲ	ⅲ	ⅲ	ⅲ	ⅲ	ⅲ	ⅱ
Humpback cells:								
8	ⅲ	ⅲ	ⅲ	ⅱ	ⅲ	ⅲ	ⅱ	−
19	ⅱ	+	−	−	−	−	−	−
24	ⅲ	ⅲ	±	−	−	−	−	−
27	ⅲ	ⅱ	+	−	−	−	−	−
33	ⅱ	+	±	−	−	−	−	−
34	ⅲ	ⅲ	+	−	−	−	−	−

This table presents evidence that the humpback varies individually with respect to an antigen with specificities related to the Ju 2 antigen of the finback. Additional evidence for a Ju-like system in humpbacks is shown Tables 5 and 6.

off San Francisco. As the reactions show, humpback cells vary individually with respect to an antigen with Ju 2-like specificities (cf. Fujino, 1958). Further studies on humpbacks collected off Okinawa have led to a tentative grouping of individuals into four types with respect to the Ju-like antigens. The first three of these types react positively to varying degrees with Ju 2 specific serums, while the fourth does not. The individuals in the different types are as follows: Type 1, K–9; Type 2, K–24, K–61, N–12 and R–82; Type 3, R–10, R–29, R–48, R–66, K–12 and K–81; Type 4, K–4 and all other individuals excepting R–45. This individual reacted positively with Ju 2 serums, but could not be studied sufficiently to decide whether it belonged to type 2 or type 3.

Table 5 illustrates typical reactions of each cell type with respect to anti-finback Ju serums. These show that a reciprocal relationship exists among the types that strengthens the argument that humpbacks vary with respect to Ju-like antigens.

TABLE 5. AGGLUTININ TITERS OF IMMUNE ANTI-FINBACK Ju SERUMS AGAINST DIFFERENT TYPES OF HUMPBACK ERYTHROCYTES FROM OKINAWA

Humpback whale cells: Type	no.	Anti-finback Ju 1 serum: Unabsorbed 40	80	160	320	640	1280	2560	5120	Absorbed by K9 cells 40	80	160	320	640
1	K 9	‡‡	‡‡	‡‡	‡‡	+	−	−	−	−	−	−	−	−
2	K24	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡	+	−	‡‡	‡‡	+	−	−
3	R29	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡	+	−	‡‡	‡‡	+	−	−
4	K 4	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡	+	+	‡‡	‡‡	‡‡	+	−

Humpback whale cells: Type	no.	Anti-finback Ju 2 serum: Unabsorbed 40	80	160	320	640	1280	2560	Absorbed by K4 cells 20	40	80	160	320	640
1	K 9	‡‡	‡‡	‡‡	‡‡	‡‡	‡‡	+	‡‡	‡‡	‡‡	‡‡	+	+
2	K24	‡‡	‡‡	‡‡	+	−	−	−	‡‡	‡‡	+	±	−	−
3	R29	‡‡	+	−	−	−	−	−	+	−	−	−	−	−
4	K 4	+	−	−	−	−	−	−	−	−	−	−	−	−

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That the relations among the Ju 2 positive types (1, 2 and 3) are complex is illustrated by Table 6 which summarizes the results of reactions of different cell types with anti-finback Ju 2 no. 34 serum following absorption with different type cells.

Isoagglutinins, similar to those described by Fujino (1952), were easily recovered in the supernatants of dialysed samples at approximate dilutions of one in two from whole blood. These could be used directly, or freed from excessive hemoglobin through salting-out by one third saturation with ammonium sulfate. The relationship of isoagglutinins to antigenic types is shown in Table 7. These parallel the results of Ju 2

TABLE 6. THE REACTIONS OF DIFFERENT CELL TYPES WITH ANTI-FINBACK Ju 2 No. 34 SERUM ABSORBED IN VARIOUS WAYS

a) Cross-absorption reactions, serum dilution one in ten

Absorbing cells: Type	no.	Test cells: K 9	N12	K24	K61	R10	R29	R48	K 4
1	K 9	–	–	–	–	–	–	–	–
2	N12	###	–	–	–	–	–	–	–
2	K24	###	–	–	–	–	–	–	–
2	K61	###	–	–	–	–	–	–	–
3	R10	###	##	##	##	–	–	–	–
3	R29	###	##	##	##	–	–	–	–
3	R48	###	##	##	##	–	–	–	–
4	K 4	###	###	###	###	##	##	##	–

b) Agglutinin titers after progressive absorptions

	Test cells: Type	no.	Dilution of serum: 10	20	40	80	160	320	640	1280
Absorbed by K4 cells	1	K 9	###	###	###	###	##	+	+	–
	2	N12	###	###	##	+	±	–	–	–
	2	K24	###	###	##	+	±	–	–	–
	2	K61	###	###	##	+	±	–	–	–
	3	R10	##	+	–	–	–	–	–	–
	3	R29	##	+	–	–	–	–	–	–
	4	K 4	–	–	–	–	–	–	–	–
Absorbed by K4 and R29 cells	1	K 9	###	###	##	+	–			
	2	N12	##	+	–	–	–			
	2	K24	##	+	–	–	–			
	2	K61	##	+	–	–	–			
	3	R10	–	–	–	–	–			
	3	R29	–	–	–	–	–			
	4	K 4	–	–	–	–	–			
Absorbed by K4, R29 and K61 cells	1	K 9	###	###	##	+	–			
	2	N12	–	–	–	–	–			
	2	K24	–	–	–	–	–			
	2	K61	–	–	–	–	–			
	3	R10	–	–	–	–	–			
	3	R29	–	–	–	–	–			
	4	K 4	–	–	–	–	–			

TABLE 7. THE RELATIONS OF HUMPBACK ISOAGGLUTININS TO CELL TYPES

Isoagglutinins in serum from supernatant:	Type of test cells: 1 K 9	2 K24	3 R10	3 R29	4 K 4	4 R38
K 9	–	–	–	–	–	–
K24	###	–	–	–	–	–
R10	###	+	–	–	–	–
R29	###	–	–	–	–	–
K 4	###	##	+	+	–	–
R38	###	+	–	–	–	–

The isoagglutinins in the undiluted supernatants of dialysed samples. Additional data were obtained from the other Ju 2 positive types and eight negative individuals that conformed with the observations shown above.

serum absorptions, excepting for the individual serum variations within types as shown. (One explanation for this variation simply could be differences in titers of the antibody fractions concerned).

As in the finback whale, natural antibodies were found in the serums of various animals that reacted positively with cells showing Ju 2-like specificities (Table 8). As far as studied the reactions of these serums appears to be similar to that of the immune serums and isoagglutinins already described, and shows that normal serums will provide a useful source of antibody for further research.

TABLE 8. INDIVIDUAL VARIATIONS IN THE REACTIONS OF HUMPBACK ERYTHROCYTES WITH NORMAL ANIMAL SERUMS

Okinawa sample

Humpback erythrocytes:		Pig				Sheep-1				Sheep-2			
Type	no.	4	8	16	32	4	8	16	32	4	8	16	32
1	K 9	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺
2	N12	⧺	⧺	+	−	+	±	−	−	⧺	+	−	−
3	R48	−	−	−	−	−	−	−	−	−	−	−	−
4	R14	−	−	−	−	−	−	−	−	−	−	−	−

		Horse (unabsorbed)							Horse (absorbed with R-45 cells)						
Type	no.	8	16	32	64	128	256	512	8	16	32	64	128	256	512
1	K 9	⧺	⧺	⧺	⧺	⧺	⧺	+	⧺	⧺	⧺	⧺	⧺	⧺	+
2	K24	⧺	⧺	⧺	+	−	−	−	⧺	⧺	±	−	−	−	−
3	R48	⧺	+	−	−	−	−	−	−	−	−	−	−	−	−
4	K 4	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2 or 3	R45	⧺	⧺	⧺	±	−	−	−	−	−	−	−	−	−	−

San Francisco samples

	Sheep (unabsorbed)						Sheep (absorbed with 1 cell)					
No.	2	4	8	16	32	64	2	4	8	16	32	64
1	⧺	+	±	±	−	−	−	−	−	−	−	−
8	⧺	⧺	⧺	⧺	+	±	⧺	⧺	⧺	+	±	−
15	⧺	⧺	+	±	−	−	+	+	−	−	−	−
28	⧺	⧺	+	±	−	−	+	±	±	−	−	−
29	⧺	⧺	⧺	+	+	±	⧺	⧺	±	−	−	−

This table shows the potential usefulness of normal animal serums in population studies on humpbacks.

While not enough work has been done to establish a genetic hypothesis explaining the interrelations of humpback blood types, it is obvious that more than two Ju-like alleles are involved, and probable that these can exhibit both quantitative and qualitative differences with respect to each other.

Table 9 shows that even in the present state of our knowledge the humpback Ju antigens provide us with genetic markers that will be very useful in population research.

TABLE 9. THE RELATIVE FREQUENCIES OF OCCURRENCE OF DIFFERENT CELL TYPES IN THE POPULATION OF HUMPBACK SAMPLES FROM OKINAWA

	Type				unclassified	not tested (hemolysed)	total no. of samples
	1	2	3	4			
No. of samples of each type	1	4	7	87	1	5	105

TABLE 10. SUMMARY OF TEMPORARY CLASSIFICATION OF HUMPBACK BLOOD TYPES AS DETERMINED BY FINBACK JU SPECIFICITIES

		Type of test cells:			
		1	2	3	4
Type of absorbing cells:	1	−	−	−	−
	2	+	−	−	−
	3	+	+	−	−
	4	+	+	+	−

Additional species.

Two samples of sperm whale (*Physeter catodon*) blood from San Francisco, and one of the True's Porpoise (*Phocaena dallii truei*) from northern Japan have been collected and successfully recovered. Studies on the preservation of fur seal (*Callorhinus ursinus*) blood also are proving successful. Recently Suzuki et al. (1958) have reported successful use of the glycerol-freezing technique in the investigation of antigenic differences in tunas. They have evidence, from cells collected in this manner, that shows population differences between the blood types of albacore from the Pacific and Indian Oceans.

DISCUSSION AND SUMMARY

The above survey shows that it is possible to adapt on a large scale the glycerol-freezing method of erythrocyte preservation as an aid in whale blood type studies. The method is simple and well suited to the rough conditions of field collection, providing refrigeration and freezing equipment is available, and reasonable care is taken by the collector. While the technique has proven adequate in its present form, further research is desirable in order to control hemolysis, determine optimum sample size, examine the effectiveness of ethylene glycol and related compounds etc. (cf. Strumia et al., 1958; Stone, 1957). Attention is called in this regard to the facts that some species of fish vary with respect to the relative fragility of their erythrocytes (Cushing, Ridgway, Suzuki, unpublished data), that marine teleosts are variable with respect to the isotonicity of their blood (Green, 1953) and that the erythrocytes of some fishes are remarkably sensitive to traces of silver ion in sodium chloride solutions (Ball, 1933).

The usefulness of the glycerol freezing technique is illustrated in several specific ways in the present study. These include the accumulation of additional data on the frequencies of Ju blood types in finback whales, the discovery of antigens in humpback whales that are closely related to the Ju antigens of finback whales, and the demonstration of antibodies in the serums of various domestic animals that are highly specific for the Ju 2 type antigens in both whale species.

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