drome in rats, may suggest that the use of choline as a supplementary aid in the treatment of various types of anemia should be further explored. Some of the clinical results referred to above indicate that it may be of particular value in cases complicated with liver damage. Further investigations on the nutritional implications of this interrelationship are underway in this laboratory.

Summary. 1. The incidence and severity of renal injury in weanling rats fed diets low in choline and methionine were markedly decreased by supplementing the diet with a vitamin B_{12} concentrate or crystalline vitamin B_{12}.

2. Under the conditions of these experiments, 30 μg of vitamin B_{12} per kg of diet could replace about one-half of the supplementary choline or methionine required for protection against kidney damage.

3. When sub-protective levels of choline were fed, the addition of vitamin B_{12} caused a significant increase in weight gain. However, when an adequate protective level of choline was fed, no increase in weight gain was obtained from the addition of the vitamin.

4. The results established the existence of an interrelationship between vitamin B_{12}, and choline or methionine.

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17131. Relation of Oxygen and Temperature in the Preservation of Tissues by Refrigeration.

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An inquiry has been made into conditions which may influence the viability of 1x1 cm areas of biopsied rabbit skin during refrigeration at 0° and 6-8°C. Since the availability of oxygen, as well as the nature of the storage medium, had an important influence on the preservation of viability at these two temperatures, these relationships may be of interest to those wishing to store or ship tissues for surgical and other purposes.

Tissues separated from the circulation rapidly become anoxemic and necrotic at room or body temperatures. This condition may be prevented in uterine or intestinal strips by oxygenation or by chilling.[1,2] The survival of ligated limbs[3] and of the cells in whole embryos[4,5] or organs[5,6] is optimal (among the widely spaced temperatures which have been studied) at 0°. At this temperature respiration is minimal, while oxygen solubility in water is twice that at 30°.

Though Lambert,[7] Carrel,[8] and Hetherington and Craig[5] found 0 to 7° favorable for preserving the small masses of crowded cells in embryonic tissue fragments, there is considerable evidence that the thin perimeter of migrating and dividing cells in established tissue cultures has maximal longevity around 30°[9,10] and are unable to re-establish growth after refrigeration for a few days.[9,11-13] Upon considering the fact that large tissues are

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11 Carpenter, E., *J. Exp. Zool.*, 1945, **98**, 79.

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killed more rapidly at higher temperatures and small groups of thinly spread cells at low temperatures, it seemed not unlikely that one of the common denominators might be a question of oxygen supply and demand.

Methods. After shaving and scrubbing, the chosen area of rabbit skin was covered for 2 minutes with a wet pack of tincture of iodine diluted 1:2 in water, and again scrubbed with alcohol. The strips of biopsied skin, either full- or half-skin depth were cut into 1x1 cm pieces. One piece of skin was explanted immediately and the others (usually 8) were stored.

The tissues were stored in two types of containers: a) 13 mm test tubes with just sufficient (0.2 cc) mineral oil or 10% rabbit serum to cover tissues, and also in such tubes filled with 9 cc of these fluids in order to replace the atmosphere, or b) in 6 cc screw cap vials suitable for mailing. These vials were packed approximately ¾ full of washed Pyrex glass wool and a second (removable) plug was added to fill the remaining space. One cubic centimeter of sterile medium moistened the entire surface of the fibers by capillarity, leaving about 4.5 cc of air space, while 5.5 cc fluid completely displaced the atmosphere. Penicillin and streptomycin at 100 units per ml. and sulfadiazine at 20 mg% were at times included in the storage media.

Tissue culture reagents. 1. Balanced salt solution (BSS): One liter of concentrated stock stored at room temperature with 4 cc chloroform: NaCl, 80 g; KCl, 4.0 g; $MgSO_4 \cdot 7H_2O$, 2.0 g; $CaCl_2$, 1.4 g (dissolved separately); $Na_2HPO_4 \cdot 2H_2O$, 0.6 g (1.5 mg of $Na_2HPO_4 \cdot 12H_2O$); KH_2PO_4, 0.6 g; glucose, 10.0 g. To this is added 100 cc of phenol red 0.2%. This stock solution was diluted 1:10 (Ca = 5 mg %, P = 2.4 %) and autoclaved in 20 cc amounts in screw cap Pyrex bottles. One half cubic centimeter of autoclaved 1.4% (isotonic) $NaHCO_3$ was added to each bottle and the solution stored in the refrigerator for CO_2 equilibration to pH 7.6 before final tightening of the caps.

2. *Chicken plasma* (*CH*) Cock blood was drawn into a 10 cc syringe containing 0.2 cc of 10% sodium citrate, centrifuged twice and stored in 1 to 2 cc amounts in plain glass tubes at –20°C.

3. *Beef embryo juice* (EM_{50}): Three- to 5-inch beef embryos were emulsified in a Waring blendor for 1.5 minutes with an equal volume of BSS. The suspension was centrifuged at top speed for 30 minutes and the supernate respun in cotton balltubes.

4. Rabbit serum fresh, or frozen at –20°C.

Efficient sterility tests were provided by having a small fluff or ball of cotton in all tubes used for the second centrifugation. Clean separation of the supernate from the sediment was assured, while approximately 99% of the possible contaminating organisms remained below the packed cotton. Brewer's medium was pipetted onto the cotton and the sediment to a depth of approximately 3 inches and the whole incubated at 31° for one week.

Tissue culture methods. Both freshly biopsied and stored skin* were sampled for viability after being cut into 2x2 mm fragments in Brewer's medium, which was pipetted off for sterility control. Twelve of the fragments from each piece of skin were selected at random, 3 being transferred to each of 4 tubes previously coated with one drop of chicken plasma. After orientation of the explants and mixing the three drops of medium (see liquid phase) with the plasma, the tubes were laid horizontally for coagulation. One cubic centimeter of liquid phase (rabbit serum 20%, embryo juice 2%, penicillin and streptomycin 100 units each and sulfadiazine 20 mg %) was added to each tube prior to stoppering and slanting for incubation at 37° for one week.

At the end of one week, data were collected on the proportion of explants showing positive growth and on the area of new growth around the explants. Epithelial and fibrocytic outgrowth was distinguished and each rated on a scale of 1 to 4 plus, 1+ being assigned to explants with sufficient cells to surround only one of the four sides and 4+ to those completely surrounded.

* The removal of mineral oil from stored tissues by shaking them in several replacements of ethyl ether did not influence their viability.

TABLE I.
Survival of Biopsied Skin at Low Temperatures.

Exp. No.	Type of biopsy	Explanted immediately				Storage		Survival at 1 week				Survival at 2 weeks			
		% Pos.	New area (mm^2)	Cell* rating E	Cell* rating F	Temp. °C	Medium	% Pos.	New area (mm^2)	Cell rating E	Cell rating F	% Pos.	New area (mm^2)	Cell rating E	Cell rating F
1	Full depth	100	16	2.3	3.4	8	Min. oil full	67	19	1.0	1.5	0			
							Min. oil 0.2 cc	100	35	1.3	3.7	8	0		
							RS_{10}† full	100	8	1.3	2.9	58	1.3	.2	.3
							RS_{10} 0.2 cc	100	35	1.5	3.2	100	35.0	2.8	3.7
2	Full depth	100	24	0.5	3.1	0	Min. oil full	42	2	0.5	1.4	0			
							Min. oil 1 cc	0				0			
							RS_{10} full	92	9	1.0	2.7	17	0	0	0.05
							RS_{10} 1 cc	83	6	1.1	2.3	8	0	0	0.01
3	Full depth	100	16	0.1	3.6	0	RS_{10} full	92	15	0.3	3.0				
							R blood (1 cm)	92	15	0.1	2.3				
	Tiersch graft	100	16	0.4	3.2	0	RS_{10} full	83	8	0	1.9				
							RS_{100} full	100	16	0	2.8				
							R blood (1 cm)	100	16	0	2.7				
							R blood (3 cm)	67	6	0	0.8				

* Epithelial (E) and fibrocytic (F) outgrowth from each explant were rated on the 4+ scale. Cell ratings are average values from each group of 12 cultures. Since one side of the explants was covered with epidermis, 1+ or 2+ tends to be a maximal value for E and 3+ a maximal value for F during early outgrowth.

† Rabbit serum 10%, RS_{100} = Rabbit serum 100%.

Results. Representative results are summarized in Table I. During refrigeration at 6-8°, the exclusion of atmosphere was always detrimental (see first experiment). In tubes or vials filled with mineral oil the blood in the vessels became blue, and the viability declined in less than one week. No cells could be cultivated after two weeks. In tubes filled with rabbit serum 10% the viability declined steadily over the two week period. The pH indicator in the upper stratum of the liquid retained its original color while the fluid nearer the tissues became strongly acidified within one week.

With just enough oil to cover the skin and prevent evaporation, the blood in the vessels remained red. Viability was maintained at a high level for one week, but declined sharply during the second week. In shallow layers of rabbit serum 10% the pH fell to 6.8 or 7.0 within one week but in spite of the small reserve of medium and of buffer the viability did not decline within two weeks.

Preservation of viability at 0° proved to be far less satisfactory in any medium employed, and particularly in the presence of atmosphere (see second experiment). No biopsies survived for one week in 0.2 to 1.0 cc mineral oil. In tubes filled with mineral oil to exclude atmosphere, the results were strikingly more favorable, since approximately 50% of the cells remained alive during the first week. No cells survived after preservation in oil for two weeks. In rabbit serum 10% of the cells were more adequately supported or protected, and the results again revealed the same relationship with respect to the influence of atmosphere. In the shallower layers of medium the viability at the end of one or two weeks was only 50 to 70% of that in tubes which had been completely filled with liquid.

Since blood provides a mass of cells capable of competing for the available oxygen, this menstruum was also employed in certain of the experiments at 0°. The third experiment includes data on both Tiersch graft and full-depth biopsies removed from the same rabbit and stored at 0° for one week. It may be seen that a blood coagulum of only one centimeter in depth proved as effective as completely filling the tubes with rabbit serum 10% or 100%. Greater depths of blood (*e.g.* 3 cm) were not superior, but were sometimes disadvantageous.

The poor migration of epithelial cells in certain experiments was due to incomplete removal of the iodine used for skin sterilization (see next paper).

Discussion and summary. Although the storage of tissues at low temperature has long been recognized as a convenient means of preserving viability for relatively short periods of time, the potential advantages of refrigeration require further investigation of the nutrient materials needed to sustain metabolism and of the conditions which permit conservation of the essential enzyme systems. From the present comparison of survival in mineral oil and in dilute serum, it is evident that the usefulness of vaseline,[8,14] pliofilm[15] or moist gauze[16] as storage vehicles may be questioned. These earlier methods provide no nutrient materials and make no provision for leaching out or buffering the acids which are produced in the living tissues.

No simple rules can be stated for the control of the oxygen-temperature relationship, since it will be modified by the depth of the medium employed and by the form and mass of the tissue, and must be determined for each type and volume of tissue to be stored. For the shipping of tissues in iced containers the use of a blood coagulum may be regarded on the one hand as a convenient means of increasing the cell mass or, on the other, of providing the necessary exclusion of atmospheric oxygen.

Since blood does not permit observation of pH changes as an index of the need to replace nutrient fluids, and the blood cells must inevitably contribute to acidification and exhaustion of the medium, this menstruum is considered less desirable than a non-cellular fluid for the "banking" of tissues during hospital storage for surgical purposes. Furthermore, it is evident that during refrigeration at 6-8° any increases in nutrient and buffering

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[15] Webster, J. P., *Am. J. Surg.*, 1944, **120**, 431.

[16] Matthews, D. N., *Lancet*, 1945, **1**, 775.

fluids should be made horizontally, *i.e.*, without restricting the availability of atmospheric oxygen.

A study by the surgical staff of the Children's Hospital of the preservation of aortic segments for surgical repair of large blood vessels has produced results in accordance with these principles. Their publication[17] describes the conditions under which blood vessels may be maintained in 10% homologous serum for as long as 50 days.

In conclusion, 10% serum is superior to mineral oil as a refrigeration menstruum because it provides nutrient and dilutes or buffers the acids which result from metabolism. At 6-8° oxygen is required to support metabolism, while at 0° viability is extended by completely filling the storage tubes with liquid or by covering the tissue with a blood coagulum.

[17] Peirce, E. C., Gross, R. E., Bill, A. H., and Merrill, K., *Ann. Surg.*, 1949, **129**, 333.

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17132. Sterilization of Skin.

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It is frequently stated that in the strict bacteriological sense it is impossible to sterilize skin. This concept apparently arises from the use of methods which do not insure that an appropriate disinfectant is applied in active form for an adequate interval of time. Since the rich and complex nutrients used for cell cultivation provide an excellent pabulum for a variety of microorganisms, long experience in sterilization of leprous and normal skin prior to biopsy for tissue culture induces us to present evidence that skin sterilization is not a difficult problem.

Early attempts to sterilize well scrubbed skin by painting with tincture of iodine were at times successful and at times failures. The inconstancy of the results suggested the desirability of controlling the action of iodine with respect to concentration and time of action. By covering the area to be biopsied with a heavy patch of fabric, dripping iodine solution (tincture of iodine 1:2 in 70% alcohol by weight) on this patch until it was saturated, and then removing the patch and recleaning with alcohol after an interval of 5 minutes, the occurrence of scattered contamination was terminated. During the next several years it was noted that epithelial cells rarely if ever migrated from fragments explanted from the papillary or uppermost layer of the skin, while abundant epithelial cells appeared around fragments explanted from the deeper or reticular layer of the skin.

Upon the first occasion when a biopsy was taken after painting the skin with 2% Mercurochrome in Duponol as a wetting agent, good epithelial growth around papillary explants appeared for the first time in our experience. Nevertheless, one after another of the culture tubes gave evidence of contamination by staphylococci until 40% of the cultures had been lost. The period of applying the iodine (1:2) patch was next shortened to 2 minutes, without trouble from contamination and with the result that the superficial layer of skin produced moderate to excellent epithelial migration.

In order to inquire whether other disinfectants might be as effective as dilute iodine under these conditions and possibly less inhibitory to epithelial migration *in vitro*, three strips of skin were removed from the same individual simultaneously, one having been prepared by a two minute dressing with tincture of iodine 1:2, the second with Zephiran 1:1000, and the third with 2% aqueous Mer-