would be expected to have the same effect as hydrogenated vegetable oil used by Gyorgy and Goldblatt and the butter fat to have the same effect as lard that they used. This was found to be true in the effects of these fats on liver fat deposition(3).

Summary. 1. Rats fed on diet containing butter fat required a higher level of choline to reduce the level of liver fat to within the normal range, than did rats fed the same level of corn oil. This "requirement" for choline, as measured by liver fat, was not greater than 0.12% of choline chloride with diet containing 30% of corn oil and about 0.15% of choline chloride with diet containing 30% of butter fat. These values were not affected by supplements of cystine, or methionine and tryptophan or all 3 amino acids. 2. When no choline was fed, rats receiving butter fat grew at a more rapid rate than those receiving corn oil. It is suggested that the reduced rate of growth of rats fed corn oil was caused by kidney damage.

1. Channon, H. H., and Wilkinson, H., *Biochem. J*, 1936, v30, 1033.

2. Hartroft, W. S., *Fed. Proc.*, 1955, v14, 655.

3. Benton, D. A., Harper, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1956, v218, 693.

4. Best, C. H., Lucas, C. C., Ridout, J. H., and Patterson, J. M., *ibid.*, 1950, v186, 317.

5. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *ibid.*, 1941, v138, 459.

6. Harper, A. E., Benton, D. A., Winje, M. E., and Elvehjem, C. A., *ibid.*, 1954, v209, 171.

7. Hawk, E. A., and Elvehjem, C. A., *J. Nutr.*, 1953, v49, 495.

8. Christensen, K., *Arch. Path.*, 1942, v34, 633.

9. Hartroft, W. S., *Brit. J. Exp. Path.*, 1948, v29, 483.

10. Beeston, A. W., and Channon, H. J., *Biochem. J.*, 1936, v30, 280.

11. Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, 1940, v132, 627.

12. Gyorgy, P., and Goldblatt, H., *J. Exp. Med.*, 1949, v89, 245.

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A Technic for Cross-Transfusion of Blood in Embryonic Chicks and Its Effect upon Hatchability.* (22868)

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Although several methods for intravenous injection of chick embryos are described in the literature(1-5), none were used for purposes which required the chick to hatch after injection or withdrawal of blood. In the course of our studies on the homograft problem, it became necessary to cross-transfuse blood between 2 embryonic chicks for subsequent testing of skin graft compatibility after hatching. Trials of presently known methods for intravenous injection of chick embryos did not lead to significant survival through hatching. Most of recent technics consist essentially of locating the vessel to be injected by candling, cutting a window in the shell, putting a drop of mineral oil on the shell membrane to make the vessel visible, injecting or withdrawing blood with a 26-27 gauge needle on a syringe, withdrawing the needle, and sealing. Although this procedure seems simple, we have encountered two principal difficulties. Firstly, it is difficult to hold the needle in a small vessel for any length of time —especially if considerable amounts of blood must be withdrawn. In an attempt to solve this problem, Goldwasser and Shelesnyak(5) constructed an U-shaped egg clamp and an elaborate syringe carrier which was movable in 3 dimensions by gears. The advantage of such a stable apparatus, however, is offset by its complexity and cost of construction. The second and most important difficulty is con-

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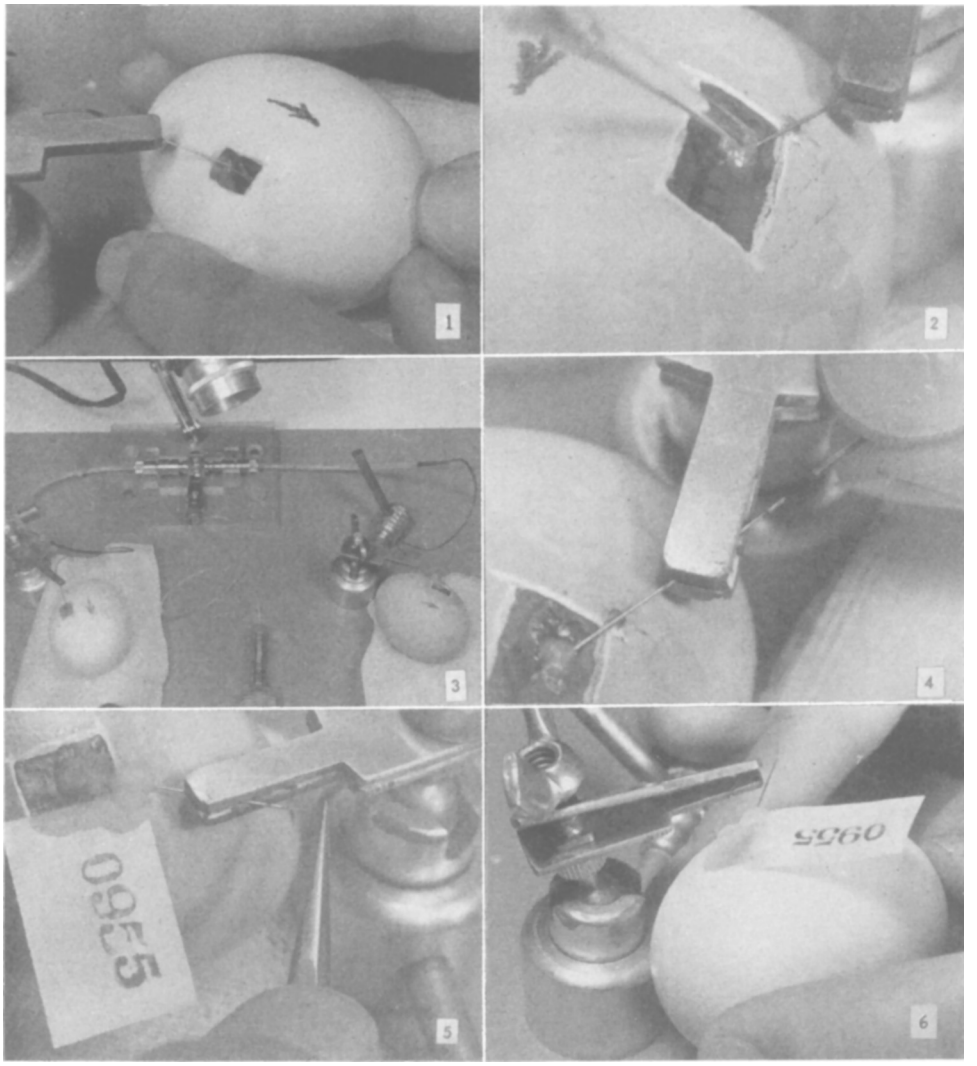
trol of hemorrhage after withdrawal of needle. Bleeding, of course, is greater the younger the embryo, since the clotting mechanism develops gradually. The effect of extensive hemorrhage becomes more noticeable when hatching of chicks is desired, since loss of blood may not be fatal immediately, but may show its effects later in development. In previous studies designed primarily for short term experiments, bleeding has been controlled in various ways: by selecting small veins for injection(4), clamping for a short period(4), withdrawing the needle slowly(2,3,5) or cauterization(1). We have tested these methods and found that none of them results in more than approximately 40% hatching. The following method has thus far yielded 82% hatching in more than 500 eggs tested.

Method. Equal numbers of White Leghorn and New Hampshire eggs were used. The eggs were put in incubator, blunt end up, and turned every 2 hours on automatic turning trays until 7th-9th days of incubation. The eggs were then incubated on their sides 2-4 days to move the main allantoic veins to the side, and uppermost side was marked. The main trunk of the allantoic vein, where chorioallantoic veins converge and enter the embryo, was then located. It is usually the largest vessel visible, and by moving the egg back and forth under a bright candler, its attachment to the embryo and to the shell can be found. The location of the vessel and direction of flow in vessel, *i.e.* toward the embryo, was marked on the eggs. A window in the shell approximately 1 x 1.5 cm was then cut with abrasive disc on a motor; care was taken not to nick the shell membrane. To stiffen the shell membrane and loosen it from the shell, mineral oil was applied to the cut edges. The shell was then taken off with a sharp needle probe and paraffin or scotch tape put over the window until the egg was ready for operation. For injection or withdrawal of blood, paraffin or scotch tape over shell membrane was taken off, and a drop of sterile mineral oil was put on window to make the vessel visible. The egg was then put on a clay mold with layer of gauze put between egg and clay to prevent adherence of excess oil on the surface of the shell. On this flexible mold, the egg was guided so that the vessel was pierced in the direction of blood flow with a 30 gauge hypodermic needle mounted on a camera tripod head (Fig. 1). To prevent any air from entering under the shell membrane, thus pushing the chorioallantoic membrane down, a drop of paraffin was immediately placed over the point of needle entry (Fig. 2). The overall apparatus for crossing blood between two eggs is shown in Fig. 3. The polyethylene tubes with the 3-way stopcock and hypodermic needle shafts were previously sterilized by soaking in aqueous solution of zephiran chloride for 24 hours. Heparinized saline was used in syringe and tubes to prevent clotting, to provide a fluid column for smooth injection or withdrawal, and to make cleaning of tubes after each injection easier. Fifteen to 40 units of heparin/ml of saline were used for 9-12-day embryos, and 50-100 units/ml for 14-18-day embryos. To prevent any intermixture of blood and saline, an air bubble was always kept between the two fluids. Any inequality in amount of blood drawn up in the 2 tubes was controlled by the three-way valve. Cross injections of blood from one egg to the other were made by detaching the polyethylene tube from the rigidly held needle (Fig. 4) and hooking it onto the other needle. Upon cross-exchange of blood, paraffin was put on the window and around the needle to hold it in place, labelled with tag, and a stylet was put in lumen of needle to make subsequent cleaning easier (Fig. 5). The egg, with needle attached, was removed from needle-holder (Fig. 6), and returned to incubator in the same position that it held during the previous 3-4 days; thus, no shearing of the needle from vessel by movement of chorioallantoic vessels could take place. In the case of 9-11-day incubated eggs, the needle was withdrawn from the vessel on 14th day, and the paraffin around the needle was pressed over the opening. In 16-18-day eggs, the needle was withdrawn 1-2 days after operation. Subsequently, the eggs were incubated in the automatic turning trays with air sac end up until 18th day, when they were put on their sides in another tray divided into



The egg is guided so that the vein is pierced by the needle (Fig. 1), and melted paraffin immediately put on shell membrane at point of needle entry to prevent entrance of air (Fig. 2). Fig. 3 shows overall apparatus when cross-transfusing blood. After withdrawal of blood the polyethylene tubes are unhooked and put on the other needle for inj. (Fig. 4). The egg is then tagged and a stylet put in the lumen of needle (Fig. 5). Finally, the egg with the needle attached is taken off the needle-holder (Fig. 6).

separate compartments. To test another method of controlling bleeding, cautery of blood vessels, with and without .04 ml of blood cross transfer, was done on 9-13-day embryos. Blood was injected and withdrawn as described. The shell membrane was then taken off, and the vessel cauterized as the needle was withdrawn. To seal the window, previously sterilized "saran wrap" plastic was laid over it and melted paraffin placed around the edges. To test the effect of transference of the air sac, the shell membrane was torn, and the window sealed as in the cauterized eggs.

Results. As is shown in Table I, 87% of 408 untreated control eggs, that were viable on 9th-11th day of incubation, hatched. Of 567 eggs with .04-0.4 ml of blood cross exchanged at 9-18 days of incubation, 82% hatched. Thus, with our method there was almost no effect on normal percentage of hatch, although 3% of hatched chicks were

TABLE I. Effect of Intravenous Cross-Transfusion during Embryonic Stages upon Hatchability of Chicks.

ml of blood cross-transfused	Incubation, days	No. hatched/No. done	No. aborted at: 18-21 days incubation	14-18 days incubation	10-14 days incubation
.04	9	29/ 34	5	0	0
	10	52/ 68	11	3	2
	11	24/ 29	2	1	2
	Subtotal	105/131 (80%)	18 (14%)	4 (3%)	4 (3%)
.1	10	26/ 30	4	0	1
	11	11/ 16	2	0	3
	13	6/ 9	2	1	0
	Subtotal	43/ 55 (78%)	8 (14%)	1 (2%)	4 (7%)
.1 (twice)	10	19/ 21 (90%)	2 (10%)	0	0
.2	10	15/ 18	3	0	0
	11	127/148	13	2	6
	12	8/ 8	0	0	0
	18	8/ 9	1	0	0
	Subtotal	158/183 (86%)	17 (9%)	2 (1%)	6 (3%)
.2 (twice)	10	19/ 21	2	0	0
	11	76/ 97	14	2	5
	16	13/ 17	4	1	0
	Subtotal	108/135 (80%)	20 (15%)	3 (2%)	5 (4%)
.4	14	16/ 21	1	1	3
	16	10/ 11	0	1	0
	17	7/ 10	2	1	0
	Subtotal	33/ 42 (79%)	3 (7%)	3 (7%)	3 (7%)
	TOTAL	466/567 (82%)	68 (12%)	13 (2%)	22 (4%)
Controls—No previous treatment		355/408 (87%)			
Inj. into arteries		16/ 19 (84%)	3 (16%)	0	0
Cautery of vein, with or without blood transfer		23/ 64 (36%)	21 (33%)	3 (5%)	17 (27%)
Air sac transferred to side on 11th day		19/ 32 (59%)			
Air sac transferred to side on 17th day		21/ 26 (81%)			

lame or otherwise sickly at hatching. All other chicks were healthy, showing no outward effects of the blood transfer. Over 200 of these chicks have been kept and observed for 3-9 months. Of the aborted eggs, most deaths occurred shortly before hatching (12%). Some deaths occurred after 14-18 days incubation (2%), and some shortly after cross injection (4%).

It should be mentioned that these eggs represent the results of 34 consecutive separate experiments since the development of this technic. Eggs of only one experiment were not included in the Table. Due to accidental failure of incubator humidity control, the humidity dropped to 47% when the eggs had been incubated 11-13 days. In this experiment, 9 out of 11 died shortly after the operation and only one hatched.

The percentage of chicks hatched did not vary significantly with withdrawal and injection of .04-0.2 ml of blood in eggs incubated 9-18 days. Nor did the per cent mortality differ with the cross-transfer of 0.4 ml of blood in 14-17-day-old eggs. The injection and withdrawal of 0.2 ml of blood once and twice, resulted in 86% and 80% hatch rate, respectively. Also, as can be seen in Table I, incubation age at which blood is cross-transferred (between 9th and 18th day) does not seem to affect hatching in any significant way.

Insertion of needle into arteries had very

little effect on hatchability of eggs, since 16 out of 19 (84%) hatched. However, of the 16 hatched, 5 (31%) were lame. As mentioned previously, when the needle was inserted, presumably in the direction of flow into veins, only 3% were lame. It is possible that these lame chicks may have resulted from inadvertent injection in opposition to the flow in the vein.

Withdrawal of the needle just after injection, followed by cautery of blood vessels, produced only 36% hatch in the 64 eggs tested (Table I). About a quarter of the eggs so tested died shortly after cauterization, and a third died just before hatching. Because cauterization involves removing the shell membrane and consequent shifting of air sac to an abnormal region, the effect of tearing the membrane was tested on eggs. As shown in Table I, when this procedure was used, 59% of the eggs operated upon on the 11th day hatched, thus accounting for much of the mortality due to cautery. Tearing the shell membrane on 17th day of incubation, however, had hardly any effect since 81% of these eggs hatched.

Discussion. With the present technics, 82% of intravenously cross-transfused chick embryos have hatched as compared to 87% of controls. The advantages of this technic as far as obtaining a large percentage of hatchability are the following: 1) Almost no bleeding occurs. This has been accomplished by leaving the needle in the vessel until clotting mechanisms of the blood are more fully developed and until the vessel tended to produce scar tissue around the needle. Other methods of controlling bleeding have usually depended upon selection of small vessels, but when fairly large amounts of blood must be withdrawn, the task of withdrawing blood from small, thin-walled vessels is a tedious one. If medium or large-sized vessels were used, clamping of vessels or slow withdrawal of needle did not prevent hemorrhage. Cauterization of vessels produced only 36% hatchability. 2) The rigid needle support makes piercing of blood vessel relatively simple. It also keeps the needle stable, thus permitting slow injection or withdrawals. 3) For cross-transfusion of blood, the vessel need be pierced only once both for removal and injection of blood. 4) By not removing the shell membrane, the air sac is left intact. Leaving the air sac in its natural position at the blunt end seems to be important for the normal hatching of eggs. If the air sac was transferred to the side on 10th-11th day of incubation only 59% hatched as compared to 87% of control eggs. 5) A minimum of sterile technic is necessary, since the contents of the egg are not exposed to the exterior except at point where needle is inserted through the shell membrane. 6) The method is a fairly rapid one; the cross-transfusion of 0.2 ml of blood between 2 eggs can be done in 5-10 minutes. 7) The method permits multiple bleeding or injection during the course of incubation, since the needle is not removed, and clotting in lumen of needle is prevented by the stylet.

For eggs earlier than the 8th day of incubation, preliminary trials indicate that the method must be somewhat revised since the yolk sac vessels are more mobile and less firmly attached to the shell membrane. After 17th day of incubation the eggs are also difficult to work with due to development of vasoconstriction mechanisms which cause the vessels to constrict upon contact with the needle. It is important in these advanced stages to inject the large main trunk of the allantoic vein.

Summary. A method is described by which .04-0.4 ml of blood may be cross-transfused in 9-18-day-old chick embryos with almost no increase in mortality over untreated control eggs. Of the 567 eggs tested, 82% hatched, as compared to 87% of the control eggs.

1. Polk, A., Buddingh, G. J., Goodpasture, E. W., *Am. J. Path.*, 1938, v14, 71.

2. Eichorn, E. A., *Sci.*, 1940, v92, 245.

3. Beveridge, W. I. B., Burnet, F. M., *Med. Res. Counc. Spec. Rep. Ser.*, 1946, v256, 22.

4. Lee, H. F., Stavitsky, A. B., Lee, M. P., PROC. SOC. EXP. BIOL. AND MED., 1946, v61, 143.

5. Goldwasser, R., Shelesnyak, M. C., *Sci.*, 1953, v118, 47.

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