effort to clear them of ascites. Many will become potassium-depleted and go into hepatic coma, for potassium depletion tends to enhance any tendency which the patient has toward hepatic coma. I think these factors are all well recognized, and they all play an important role in the over-all picture.

However, there are some things which suggest that ammonia also plays an important role. First, studies using hemodialysis in an effort to clear the blood of ammonia has resulted in transitory improvements; similarly, the experiences with arginine and glutamic acid, although they are controversial, nonetheless are predicated on their role in handling ammonium metabolism. We were concentrating on the blood ammonia aspect because I think there is a difference in the patients with esophageal varices and those developing hepatic decompensation following portacaval anastomosis or with terminal hepatic failure. We did not use casein in our experiments because we tried to keep it as close as possible to the clinical situation—a patient with bleeding varices. We, therefore, elected to use whole blood for the production of ammonium intoxication. Experimentally, we have used other protein substances and found that they will also produce hyperammonium levels.

Hepatic Function After Canine Liver Transplantation

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Homotransplanted canine livers can survive in recipient dogs for as long as 3 weeks.[10,11] Evaluation of liver function and other tissue metabolism can be made during this time. Studies of this kind may lead to a better understanding of the homograft rejection mechanism.

Previous histological and biochemical studies in dogs following total homotransplantation of the liver have shown a marked proliferative cellular response in the organs and a progressive obstructive jaundice pattern in the blood. Total serum protein and albumin levels as well as albumin-globulin concentration showed no marked alterations as determined by ordinary laboratory methods.[11] When paper electrophoretic analyses were done, a fall in albumin and a rise in α_2-globulin were most characteristic.[10]

The availability of radioisotopic techniques for studying plasma protein metabolism prompted the present study with 2 objectives in mind: (1) to determine the rate at which the homotransplanted liver synthesizes individual plasma proteins carries out other liver functions, and (2) to determine if there is an alteration of plasma protein synthesis related to the homograft rejection phenomenon.

Methods

The following biochemical studies were done before and after total liver homotransplantation in 5 adult mongrel dogs surviving 4 to 16 days after transplant. Biosynthesis rates of mucoprotein, fibrinogen, albumin, α-, β-, and γ-globulins were determined using the rate of incorporation of a radioactive labeled amino acid as an index of protein synthesis. The following techniques were used as modified after Armstrong and associates.[1] Each animal was injected with 250μc. of S^{35} methionine, and serial samples of plasma and serum were drawn over a 24-hour period. The serum proteins of each sample were first separated by paper block electrophoresis into albumin, α_1-, α_2-, β-, and γ-globulins.

The radioactivity of each of the above fractions was then measured in a thin window, gas flow Geiger-Müller counter in the form of a precipitate of each fraction as previously described.[6] All re-

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sults were expressed as specific activity or counts per minute per milligram of protein and plotted against time to obtain synthesis curves (Figs. 2–4 and Table 1).

Synthesis rates of fibrinogen were obtained as follows. Each sample of plasma was treated with bovine thrombin to form a fibrin clot which was analyzed for radioactivity by a modification of the method of Jacobsson as previously reported.[4,6] The fibrin clot is dissolved and reprecipitated, and radioactivity per milligram of fibrin is determined in a gas flow scintillation counter.

Synthesis curves of the mucoprotein fraction were determined using a modification of the method of de la Heurga and associates as follows.[8] The proteins of 2 cc. of each serum sample were precipitated with perchloric acid, leaving the acidic mucoproteins in solution. These mucoproteins were then precipitated with phosphotungstic acid and their concentration determined turbidimetrically. The suspension was centrifuged, filtered, and the precipitate oxidized to a fine powder which was then suspended in a liquid medium as described by Jaffay and co-workers.[8] The radioactivity was determined in a tri-carb liquid scintillation counter.

The following liver function tests were obtained by routine clinical laboratory methods before and after transplant until the death of the animals: serum bilirubin, alkaline phosphatase, total cholesterol, cephalin flocculation, thymol turbidity, total serum protein, and serum albumin.

The donor animals were studied 4 to 6 weeks prior to transplantation to allow for disappearance of radioactivity from the tissues of the donor animals. Total transplantation of the liver was then done as previously reported.[11] Splenectomies were not performed except in one dog (No. 1); in this animal splenectomy made it easier to accomplish the transplant. Primary anastomoses were made in the arterial and venous conduits, and biliary continuity was established by a cholecystojejunostomy.

Intensive postoperative care was instituted which included blood replacement, antibiotics, and tracheobronchial aspiration as needed. When the animals were stable, eating, and required no further blood replacement, they were injected with 250μc. of S^{35} methionine on the third to fourth day after the transplant. Serial samples of serum and plasma were again drawn for the next 24 hours and analyzed for radioactivity in the same manner. Baseline samples of serum and plasma were analyzed for residual radioactivity before the injection of the S^{35} methionine, and these values were subtracted from each subsequent determination. The amount of residual radioactivity was in all instances practically negligible. Routine liver function tests were done every 1 or 2 days until the death of the animals.

Results

Survival and Clinicopathological Observations.—The 5 dogs studied survived from 4 to 16 days for a mean survival time of 9.2 days. The dog which survived 4 days (No. 5) was found dead in his cage on the fifth post-transplant day after completing the 24-hour synthesis study. Postmortem examination revealed all anastomoses to be intact, but the liver was engorged. The exact cause of death was not evident from the gross inspection of the organs. The other 4 animals survived 7, 8, 11, and 16 days and presumably

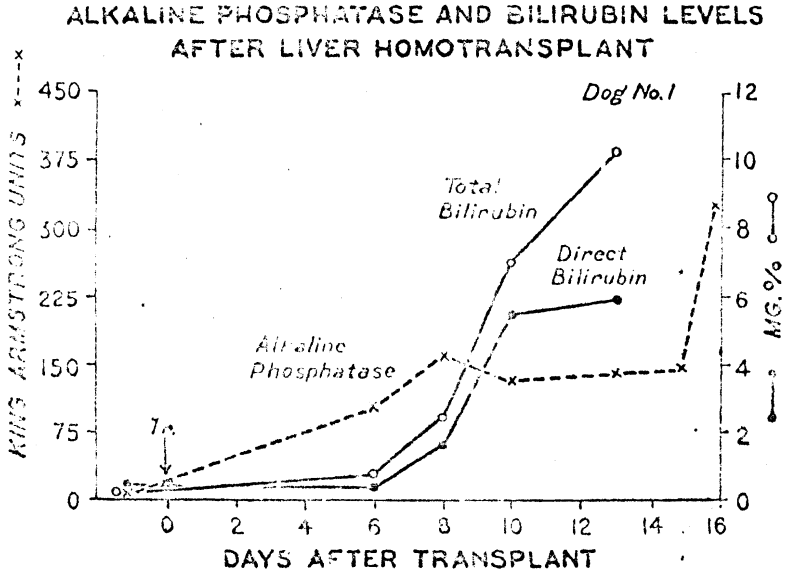

Fig. 1.—Representative results of 3 routine liver function tests as determined by clinical laboratory methods before and after the transplant procedure indicated by the arrow.

died of the consequences of graft rejection. The organs of all 5 animals showed varying degrees of changes which were previously described in a detailed histopathological study of liver transplant rejection.[11] These changes, especially of the liver, were those of a nutmeg appearance as seen in an enlarged congested liver.

Clinically the animals behaved normally after recovery from the anesthetic. Intravenous fluids including blood were given for the first 1 or 2 days, and thereafter brown sugar, bread, milk, and maple syrup were given. Physical activity was normal for 3 to 6 days. Thereafter the animals became anorectic, lethargic, feverish, and preferred to lie quietly in their cages. Intravenous glucose and saline were given to supplement their dietary intake. The animals developed leukocytosis and became jaundiced. Three of the 5 animals became comatose 12-24 hours prior to death and apparently died a toxic metabolic death similar to that seen in hepatic coma or uremia.

Biosynthesis of Plasma Proteins.—In all 5 animals there was evidence of plasma protein synthesis on the fourth day after transplant. These results are summarized in the Table and in Figures 2 to 4. In all animals fibrinogen was most actively synthesized and albumin the slowest, whether it was before or after transplant. This has previously been confirmed in other studies in normal dogs.[6] Fibrinogen is, thus, a labile protein and is

Comparison of Nine-Hour Radioactive Content of Each Electrophoretically Separated Plasma Protein Plus Fibrinogen and Seromucoid Fraction

Fraction Studied	Dog No.	9-Hr. Specific Activity Counts/Min./Mg. of Protein Before Transp.	After Transp.	Standard *t*-Test, *P* Value
Albumin	1	33	45	<0.1
	2	32	36	
	3	42	53	
	4	29	37	
	5	31	32	
α1-Globulin	—	141	255	<0.1
	—	127	172	
	—	77	231	
	—	94	134	
	—	87	205	
α2-Globulin	—	172	334	<0.01
	—	102	344	
	—	78	182	
	—	107	215	
	—	117	268	
β-Globulin	—	148	162	<0.1
	—	55	112	
	—	37	63	
	—	39	172	
	—	62	80	
γ-Globulin	—	97	215	<0.02
	—	68	178	
	—	36	78	
	—	32	124	
	—	40	138	
Fibrinogen	—	2,040	4,670	>0.2
	—	*	*	
	—	650	941	
	—	553	809	
	—	650	850	
Mucoproteins	—	*	62	<0.02
	—	32	57	
	—	20	43	
	—	23	45	
	—	22	50	

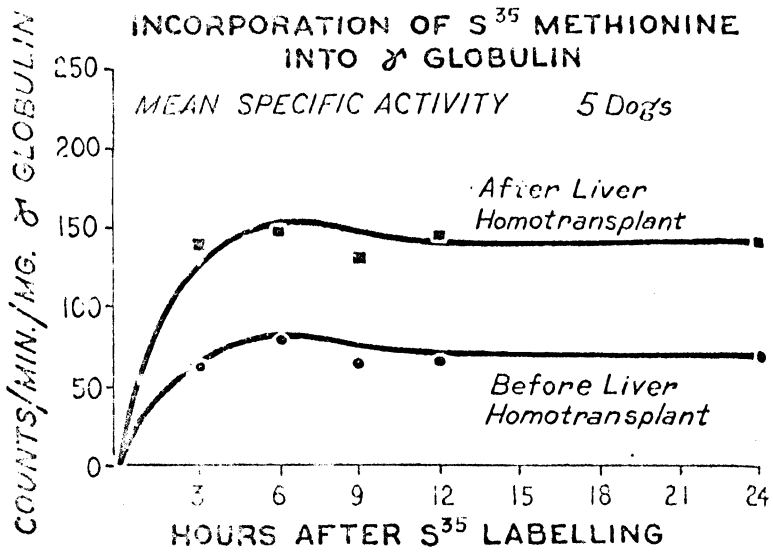

Fig. 2.—Gamma-globulin synthesis curves before and on the fourth day after total homotransplantation of the liver.

rapidly metabolized in comparison to other plasma proteins.

A comparison of formation rates before and after transplant showed the following. Albumin formation rates after transplant were increased in 3 dogs, essentially unchanged in 1 animal, and slightly decreased in another. Fibrinogen synthesis was increased in 3 of 4 animals and markedly decreased in the other. These differences are of questionable significance, though they suggest an over-all increase in synthesis in most animals. The marked decrease in fibrinogen synthesis in one dog is difficult to explain. The absence of the spleen is probably not a factor, since fibrinogen is wholly hepatic in origin.

The synthesis of α_1-globulin was increased in all animals, but in one the increase was small. In 2 animals a twofold increase was seen. Beta-globulins showed increases in 4 of 5 animals. In one animal a small decrease was noted. These differences are of borderline significance in view of the small number of animals studied.

Most significant increases were noted in the formation rates of the α_2-and γ-globulins. These fractions showed a marked increase

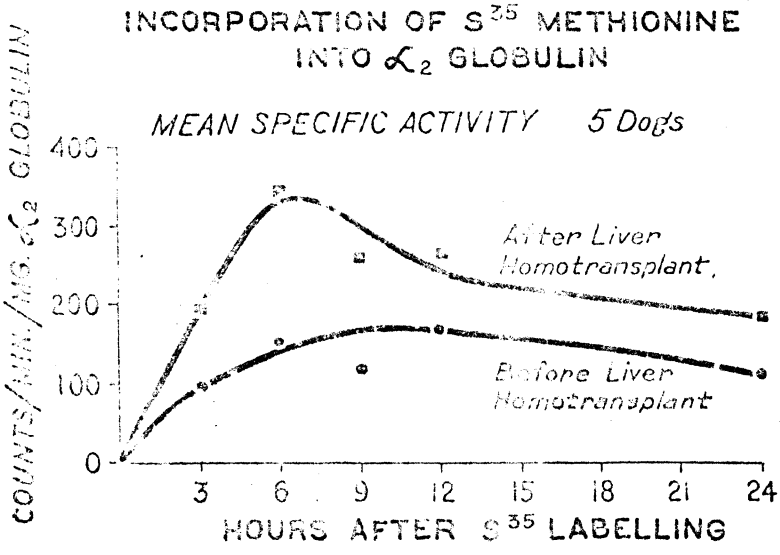

Fig. 3.—Alpha$_2$-globulin synthesis curve before and on the fourth day after total homotransplantation of the liver.

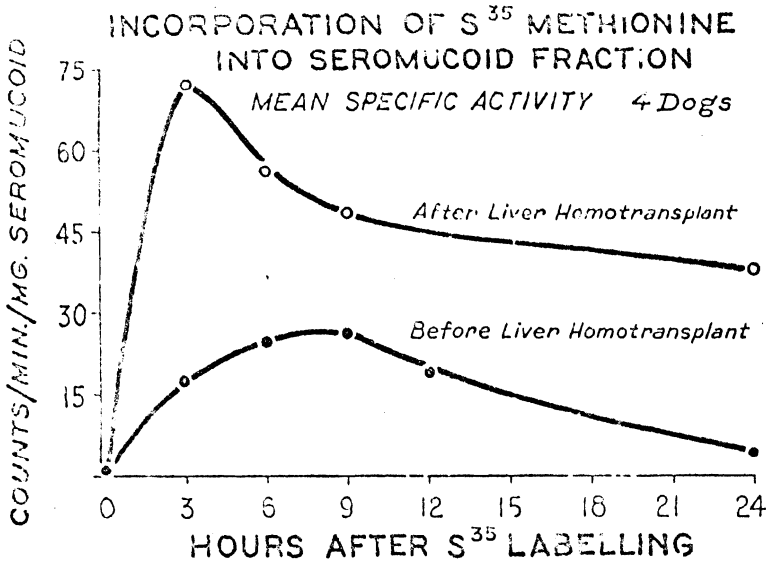

Fig. 4. — Mucoprotein (seromucoid fraction) synthesis curves before and on the fourth day after total homotransplantation of the liver.

in production in all 5 dogs. The mean increases were over 2 times normal in both fractions. In addition, the peak synthesis of α_2-globulin as noted on the 24-hour synthesis curves (Fig. 3) was attained at 6 hours after transplant as compared to 12 hours in the normal. Coincident with the increased overall formation rate of the α_2-globulin, a marked increase in synthesis of mucoproteins was noted. An earlier maximum synthesis was also seen with this fraction after transplantation as compared to the pretransplant curve. Thus the highest specific activity after transplant was seen at 3 hours in comparison to 9 hours in the normal (Fig. 4).

Liver Function Tests.—A progressive rise in *alkaline phosphatase* and *total* and *direct bilirubin* was noted in 4 animals. The fifth dog survived only long enough for the protein synthesis study (Dog 5). The alkaline phosphatase rose earlier than the bilirubin levels in 3 dogs. Both increased simultane-

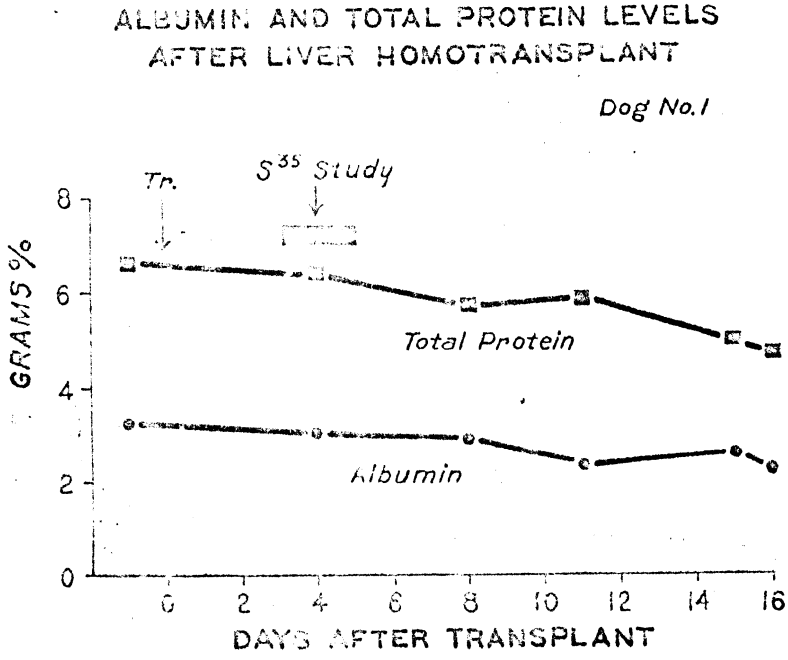

Fig. 5.—Serial determinations of total protein and albumin by routine clinical laboratory methods. Note the insignificant changes in concentration until the animal is near death. The arrows indicate the time of transplant and the plasma protein synthesis study.

ously in the fourth animal. These findings are consistent with those reported in previous studies.[10,11] *Total cholesterol* rose in 3 dogs. In 2 of these 3 animals there was a twofold increase. After an initial rise in one animal (Dog 1), there was a marked drop in total cholesterol 2 days before the death of the animal. *Thymol turbidity* increased in small amounts over baseline values in 2 animals and showed no significant changes in the third dog. *Cephalin flocculation* tests were the least consistent of all, ranging from 1+ to 3+ at various times after transplant. Dog 1 revealed a 4+ reaction on the tenth post-transplant day but varied from 1+ to 2+ before and after this.

Total protein and *serum albumin* levels, as determined by routine clinical laboratory methods, were not significantly altered except that a drop occurred before death. Thus, the total protein levels in Dog 1 varied from 6.3 to 6.0 gm. % up to the 11th post-transplant day. On the 15th and 16th day just prior to death, the total serum protein level dropped to 4.6 and 4.4 gm. %, respectively. A similar pattern was seen for serum albumin in which the terminal sample was 2.1 gm. % as compared to 3.4 gm. % before transplant, and 3.1 gm. % on the eighth post-transplant day (Fig. 5).

Qualitative changes in serum proteins by paper electrophoresis as determined by gross staining were not seen in over 50 determinations. Specific mobility measurements, however, were not determined.

Comment

The histological changes in the tissues of the host following total homotransplantation of the canine liver have been described by Moore and associates and by Starzl and co-workers.[10,11] Most striking changes occur in the organs of the reticuloendothelial system. Medawar has shown that the size of the homograft is a definite factor in the magnitude of the response elicited when skin is transplanted.[8] The larger the skin graft the more accelerated the rejection response. As Moore and associates have indicated, the liver is the largest homogenous mass of antigenic cells which can be transplanted in mammals.[10] Since the reticuloendothelial system plays a role in γ-globulin production,[9,14] the increase in γ-globulin synthesis, as seen in the present study, most likely arises from the reticuloendothelial system. This is supported by the following studies by others.

Bjørneboe has shown an intense proliferation of plasma cells in the spleen of rabbits challenged with bacterial and foreign protein antigens. These proliferative changes were correlated with increases in serum globulin which led to the conclusion that plasma cells produced most antibodies.[2] A strikingly similar plasma cell and lymphocytic proliferation was noted in many organs following total homotransplantation of the dog liver.[11] These authors concluded that the reticuloendothelial system was stimulated by the homograft rejection mechanism.[11] Additional evidence is found in homograft prolongation studies such as the one by Zukoski and associates.[16] These workers showed that an *induced* absence of the germinal centers of the lymphatic system lessened the intensity of the homograft rejection mechanism and prolonged survival of the homotransplanted kidney. The germinal centers of the lymph node and spleen are the source of the medium- and small-sized lymphocytes. Wissler and associates state in a recent review that these cells along with the plasma cells are perhaps the main cells producing antibody globulin.[14] Thus, it is apparent that cells of the reticuloendothelial system that produce γ-globulin antibody are also prominent in the role of homograft rejection. It is reasonable to assume that increased γ-globulin synthesis seen after liver transplant is caused by stimulation of the reticuloendothelial system as a result of homograft rejection. Moore and associates in their studies after liver transplant reported no increase in γ-globulin levels as measured by paper electrophoresis analyses.[10] Increases in synthesis rates, however, may not always be detected by changes in the concentration of any given protein.

The increased synthesis of α_2-globulins and mucoproteins, as seen in the present study, is most likely on the basis of a nonspecific inflammatory response which accompanies the homograft rejection phenomenon. West, Fowler, and Nathan in their work on renal homotransplants have shown that α_2-globulin was the only globulin which was consistently elevated. Increases in β- and γ-globulins were only inconsistently noted.[12] Our study showed a general increase in all globulins which may be related to the size of the antigenic stimulus, since the kidney is about one-tenth the size of the normal liver in dogs. The increase in α_2-globulin in renal transplants was further investigated by West and co-workers and found to be due to an increase in the mucoprotein fraction. These authors concluded that increase in serum mucoproteins following renal transplants was a result of nonspecific inflammatory reaction, since the same protein was elevated by the experimental production of inflammation.[12] Winzler, in a recent review, has indicated that mucoproteins are elevated in various forms of inflammation, although the exact source of these proteins is still unsettled.[13] Recent work has suggested that perhaps most of the mucoproteins are produced by the liver.[7]

Since the liver also produces all albumin and fibrinogen, it is of interest that the data presented indicate the homotransplanted liver capable of producing a normal level of formation of these proteins. In 3 of the animals, there was an increase in formation rates as compared to normal for both albumin and fibrinogen. This increase of production may represent a compensatory mechanism by the transplanted liver to replenish those proteins of hepatic origin which may be acutely depleted during the time that the recipient animal is without a liver in the course of the transplant procedure. In the case of fibrinogen, in particular, which is a protein of rapid turnover, it may also represent a response to acute depletion due to early postoperative repair and hemostasis. This may be of considerable magnitude in a surgical procedure such as homotransplantation of the liver. In addition, fibrinogen and albumin synthesis may be influenced by the inflammatory response attending homograft rejection. This is suggested by the work of Yuille and co-workers,[15] who showed an increased synthesis and turnover of albumin and, especially, fibrinogen after experimentally induced abscesses in dogs. In spite of the marked variations in plasma protein synthesis noted, the concentrations of these proteins as determined by routine laboratory methods showed no change except terminally (Fig. 5). This is perhaps explained by the fact that total protein and albumin levels do not reflect acute metabolic changes until late.

It is apparent that the plasma protein pattern following total homotransplantation of the liver shows a change from normal. Generalized increases in globulin synthesis, especially α- and γ-globulin, are related to both nonspecific and specific phases of the homograft rejection mechanism. The nonspecific changes are probably a result of the inflammatory response secondary to the rejection mechanism and are reflected primarily as increases in the mucoprotein fraction. Albumin and fibrinogen synthesis may also be related to the secondary inflammatory changes or to acute depletion in the course of the transplant procedure. Further investigation in this area is needed. Increases in γ-globulin synthesis are most probably related to direct reticuloendothelial stimulation. The magnitude of this response is probably related to the greater mass of transplanted cells. Thus, the liver, one of the largest organs which can be transplanted, evokes the most intense response.

Conclusions

Changes in plasma protein synthesis were found in dogs following total homotransplantation of the liver using isotopically labeled amino acids to determine formation rates. These changes are most likely related to a massive stimulation of the reticuloendothelial system by the rejection of a large organ such as the liver.

Increases in the globulins were most consistent. The α- and γ-globulins were synthe-

sized twice as fast after liver transplant as in the normal dog.

Increases in γ_2-globulin production were related to increased synthesis of mucoproteins. Mucoprotein synthesis rates were 3 times the normal after liver transplant. The homotransplanted liver is capable of albumin and fibrinogen synthesis on the fourth day after transplant.

A progressive obstructive jaundice is noted in the dog following liver transplantation as judged by routine liver function tests.

We express our appreciation to Mr. Joseph Sporn for technical assistance and to Dr. Richard J. Winzler for guidance in the mucoprotein analyses.

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DISCUSSION

DR. FREDERICK W. PRESTON, Chicago: Transplantation of livers into liverless dogs with subsequent survival of the animals for more than 2 weeks (one of the dogs which this group studied survived 3 weeks) is a noteworthy achievement and represents a contribution to the science of homotransplantation.

Dr. Kukral and others have shown that animals with homotransplanted livers are able to carry on most of the functions ascribed to the liver, but the report of Dr. Kukral which you have just heard presents the first detailed study of the plasma protein fractions in the animal with liver homotransplant.

Since some of the animals lived for more than 2 weeks, one might assume that the homotransplants functioned for this length of time. However, some hepatic functions are taken over by the reticuloendothelial system. The extent to which the reticuloendothelial system is able to substitute for the liver is not known. Animals with liver homotransplants make bile for the duration of their life. This function is unique to the hepatic cell and is not shared by the reticuloendothelial system.

Dr. Kukral's work does not tell us whether all of the plasma protein synthesis which he observed went on in the liver or whether the source of some of these proteins might be the reticuloendothelial

system. A totally liverless dog does not live for more than 5 days, and it usually succumbs in 36 hours or less from hepatic insufficiency and hypoglycemia. This indicates that the reticuloendothelial system is incapable of a rapid take-over of hepatic function.

In the liver-transplanted dog there may be a gradual assumption of hepatic function by the reticuloendothelial system over a period of days or weeks, and it is interesting to speculate as to whether the reticuloendothelial system may contribute to the length of survival of the liverless animal.

On the other hand, the reticuloendothelial system also has an opposite effect, in that it protects the animal's individuality by providing a mechanism for the rejection of homografts. Damaging the reticuloendothelial system with antimetabolites such as 6-mercaptopurine has made it possible for nephrectomized dogs to live for as long as 3 months following bilateral nephrectomy and renal homotransplantation, whereas without conditioning the recipient, the survival is 9 days or less—quite a difference.

Certainly one of the next steps in the field of liver homotransplantation will concern methods of conditioning the recipient. In our laboratory we have found that cytoxan is superior to a variety of other alkalating agents and antimetabolites in conditioning mice for homotransplants of skin. The homografts live about twice as long in conditioned as in control animals.

Starzl used total-body irradiation in an effort to prolong the life of some of these animals with liver transplants, but much less progress has been made in conditioning animals for hepatic than for renal homotransplants. Conditioning the recipient of a homograft may provide the key for permanent success of homografts, thus making it possible to cure disease by organ replacement.