

Fig. 2. Cytotoxic tests with H-2 antisera on C57BL/6 mouse lymphocytes and on A-strain mouse lymphocyte suspensions, mixed in various proportions by volume. The slight curvature of the two plots and their intersection near 60 percent (abscissa) rather than at 50 percent was predictable from an inequality of the cell counts of the original suspensions. The counts are highly reproducible, and the test illustrated here better demonstrates their accuracy than does a comparison with visual counts. Serum and reagents were filtered for this test.

modified dark-field optical system (9).

The absorption at 5900 Å by dead cells stained with trypan blue was greater than their scatter and caused a decrease in total light through the optical system. Living cells scattered the incident energy without significant absorption and caused an increase in the light transmitted through the optical system. Thus, the photomultiplier signals due to dead cells and living cells were of opposite polarity. The two pulse trains produced were counted on independent scalars. The scalar values were used directly as counts of live and dead cells in a sample. For most samples at least 5000 cells were counted at a rate of about 200 cells/sec.

Channels with 100- $\mu$  and 25- $\mu$  constrictions were also tested. Better signals were obtained with the smaller channel, particularly with small cells, but clogging of the channel was more frequent. This was not a difficulty with the 50- $\mu$  channel.

The accuracy of this automated method of counting was studied with a variety of cells and compared with visual counts. For the most part H-2 isoantisera were reacted with mouse lymphocytes and thymocytes, EL4 ascites leukemia cells, and Meth A ascites sarcoma cells. Even with the smallest cells, signals were adequate to distin-

guish from electrical noise; counts were reproducible within 2 percent and were paralleled with visual counts (Figs. 1 and 2).

There was a systematic error which was especially marked with small cells such as thymocytes (Fig. 1). This arose from particles that scattered sufficient light into the dark field to be registered as living cells. The error was most obtrusive as the number of dead cells approached 100 percent. At that point, machine counts indicated a residue of usually 5 to 10 percent of living cells, due to these particles. The particles which register as live cells are small, and when large cells were being counted the threshold for signals could be adjusted to exclude them; with smaller cells, this was not possible. The error was minimized by filtering the serums, Medium "199," and the trypan blue solution as described above. Corrections also can be made by obtaining background counts on an appropriate volume of serums, Medium "199," and trypan blue without cells, but we found this unnecessary after filtration. Erythrocytes should be excluded as far as possible in preparation of cell suspensions since they refract light without taking up trypan blue, and are counted as living cells.

MYRON R. MELAMED

Memorial Hospital for Cancer and Allied Diseases, New York 10021

LOUIS A. KAMENSKY\*

IBM Watson Laboratory, Columbia University, New York

EDWARD A. BOYSE

Sloan-Kettering Institute, New York 10021

#### References and Notes

1. P. A. Gorer and P. O'Gorman, *Transplant. Bull.* **3**, 142 (1956).
2. E. A. Boyse, J. Old, I. Chouroulinkov, *Methods Med. Res.* **10**, 39 (1964).
3. E. S. Curtoni, P. L. Mattiuz, R. M. Tossi, Eds., *Histocompatibility Testing 1967* (Williams & Wilkins, Baltimore, 1967).
4. A. R. Sanderson, *Immunology* **9**, 287 (1965); H. Wigzell, *Transplantation* **3**, 423 (1965).
5. L. A. Kamensky, M. R. Melamed, H. Derman, *Science* **150**, 630 (1965).
6. L. A. Kamensky and M. R. Melamed, *Ann. N.Y. Acad. Sci.*, in press.
7. F. Celada and B. Rotman, *Proc. Nat. Acad. Sci. U.S.A.* **57**, 630 (1967).
8. R. O'Brien and P. Gottlieb, *J. Histochem. Cytochem.*, in press.
9. An Osram XBO-75 xenon source was used with Koehler illumination of a 50- $\mu$  constriction in the flow channel. A 2.8-mm, 0.72 N.A. Polaroid Grey (10) reflecting objective lens (Bausch and Lomb) imaged the flow channel onto an aperture, limiting the object field to a 50- $\mu$  square. The back focal plane of the objective was imaged through a 5900-Å interference filter onto a Fairchild-2485 photomultiplier tube. The aperture of the Carl Zeiss UV-Kondensator (Ultrafluor; 0.8 N.A. with iris diaphragm) was adjusted so that all but about 25 percent of the entrance cone of light to the objective was obscured by the central mirror of the reflecting system.

10. D. S. Grey, *J. Opt. Soc. Amer.* **40**, 283 (1950).

11. We thank C. Iritani for performing the cytotoxic tests. E.A.B. was supported in part by National Cancer Institute grant No. CA-08748 and a John A. Hartford Foundation, Inc. grant.

\* Present address: Biophysics Systems, Inc., Katonah, New York 10536.

4 September 1968

### Human C'3: Evidence for the Liver as the Primary Site of Synthesis

Abstract. *The liver is the primary, if not sole, site of synthesis of the third component of human complement, as shown by a change in the recipient from C'3 FS<sub>0.6</sub> to C'3 SS, the donor type, following homotransplantation of the liver.*

Polymorphism in the plasma proteins has provided useful markers in determining their source in homotransplantation of the human liver. If a given protein is of different type in liver donor and recipient, and if the recipient's type changes to that of the donor after transplantation, the liver must be the primary, if not sole, site of production of that protein. Such a change in type has been demonstrated for haptoglobin (1) and more recently for Gc-globulin (2). Experiments in animals, which utilize incorporation of radioactive labels (3) and fluorescent antibody localization (4), have shown that haptoglobin is indeed synthesized by the liver.

With the recognition of allotypy in the third component of human complement (C'3), the question of hepatic synthesis of this protein can be examined in similar fashion. There are seven known alleles for C'3 (5) and the various gene products are identified by differences in electrophoretic mobility. In Caucasians, the C'3 S (slow) allele has an approximate gene frequency of 0.75, while the F (fast) gene has a frequency of about 0.25. The other allotypes, F<sub>1</sub>, F<sub>0.8</sub>, F<sub>0.5</sub>, S<sub>0.6</sub>, and S<sub>1</sub>, are relatively rare.

Typing of C'3 was performed by prolonged agarose electrophoresis of fresh serum or serum promptly separated from clotted blood and stored at -80°C as previously described (5); and typing of haptoglobin, by the method of Smithies (6) with starch gel electrophoresis and a discontinuous buffer system (7). Quantitation of C'3 was carried out with the electroimmunodiffusion technique of Laurell (5, 8) or a nephelometric method (5, 9) or both,

with an antiserum to purified C'3 prepared in this laboratory by the method of Nilsson and Müller-Eberhard (10). This antiserum was monospecific when reacted with whole human serum in immunoelectrophoresis (11). Antigen-antibody crossed electrophoresis was performed as described by Laurell (12) with this antiserum to human C'3.

The liver recipient was a 16-year-old white male with an unresectable hepatoma grossly confined to the liver and a few portal nodes. There was no other liver disease either clinically evident or visible on routine histologic examination. The liver donor was a previously healthy 12-year-old white male who sustained fatal cerebral damage in an automobile accident. Orthotopic transplantation of the liver was performed, the recipient receiving 5 liters of whole blood during the operation.

Following transplantation of the liver, the concentration of C'3 in serum fell from 207 mg/100 ml to a low of 41 mg/100 ml on the 4th postoperative day. Thereafter, the concentration gradually rose to a normal value of 135 mg/100 ml by the 14th postoperative day. From the 3rd to the 6th postoperative weeks, during which time the patient had evidence of bacterial infection, the C'3 concentration fluctuated between 101 and 37 mg/100 ml. One day prior to death from pulmonary sepsis, 45 days after homotransplantation of the liver, the C'3 concentration was 28 mg/100 ml.

Typing revealed that the liver recipient was C'3 FS<sub>0.6</sub>, while the donor was C'3 SS; S<sub>0.6</sub> is an uncommon allotype (5, 13) but, since the recipient's mother was C'3 SS<sub>0.6</sub>, any influence of the hepatoma on the C'3 type can be eliminated. The C'3 type of the donor, SS, is the most common in all populations studied thus far (5).

Figure 1 shows, from left to right, the C'3 patterns of the donor, the recipient prior to operation, and the recipient at 20 hours and at 6 days after the operation. The recipient's C'3 type changed to that of the donor following transplantation of the liver, as seen in the sample obtained 6 days after the operation. The 20-hour sample shows traces of C'3 FS<sub>0.6</sub>. This is compatible with previously published observations on the metabolism of human C'3 in which the fractional catabolic rate in normal subjects was found to be 1.3 to 3.4 percent of the plasma pool per hour (14), with a corresponding biological half-life of 24 to 72 hours. Samples obtained up to 6 weeks postoperatively

Table 1. Serum protein types of liver donor and recipient before and after homotransplantation. Hp, haptoglobin; Tf, transferrin; and Gc, Gc-globulin.

Subject	Types of serum protein			
	C'3	Hp	Tf	Gc
Donor	SS	2-2	CC	2-1
Recipient, prior to transplantation	FS <sub>0.6</sub>	2-1	CC	2-1
Recipient, 6 days after transplantation	SS	2-2	CC	2-1

showed only C'3 SS. The typings were confirmed by antigen-antibody crossed electrophoresis. The minor gene product of C'3 F (5) disappeared from the recipient's serum and was replaced by the C'3 S minor product.

Two reasons for the change in C'3 type were considered. The possibility that the recipient's C'3 was derived from the transfused blood (which would be expected to contain more C'3 SS

than other types) was unlikely because the recipient's concentration of C'3 was highest and was of type SS when he had received no blood for 11 days. A second explanation for the change in C'3 type involves the agents the recipient received to suppress rejection of the transplanted liver. These included azathioprine, prednisone, and globulin from horse antiserum to human lymphocytes. We therefore examined serums from donor and recipient of a kidney, since the recipient was on the same course of treatment to suppress rejection. The kidney donor was C'3 SS while the recipient remained C'3 FS following transplantation and immunosuppression.

The recipient's plasma proteins before and after liver transplantation are given in Table 1. The recipient's haptoglobin changed from Hp 2-1 to Hp 2-2 as reported previously by others. (1). The types of Gc-globulin and transferrin were the same in recipient and donor.

These observations indicate that the liver is the primary, if not sole, site of synthesis of C'3 in man. Previous work,

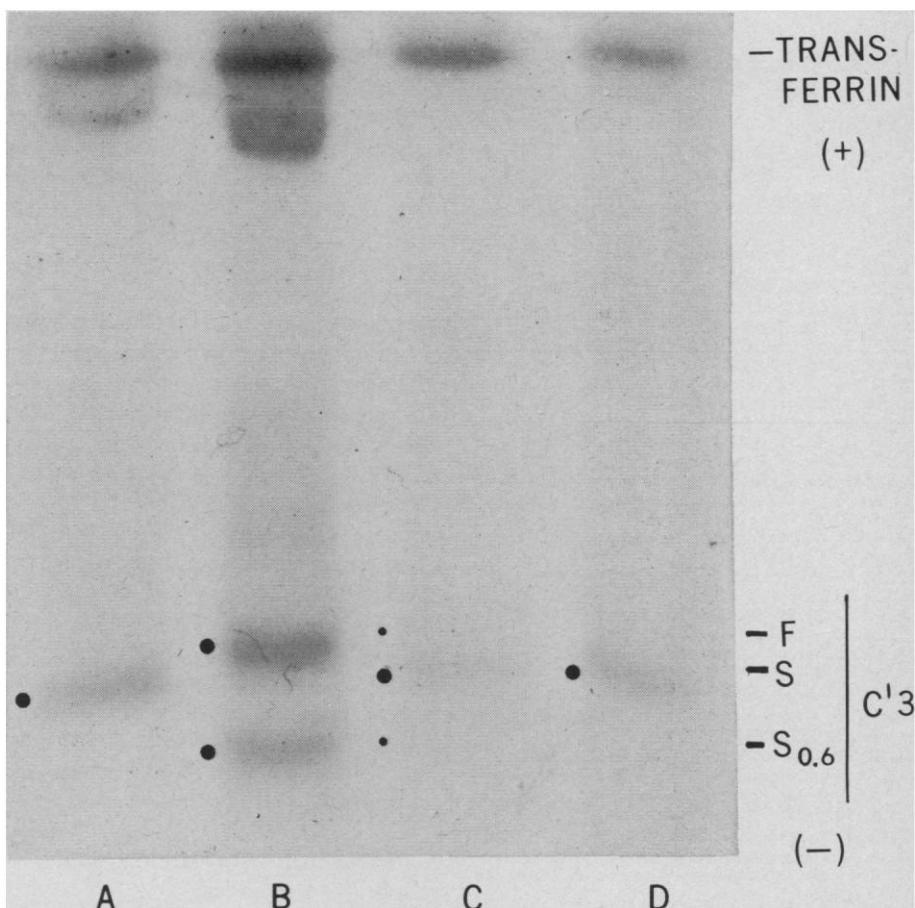


Fig. 1. Prolonged agarose electrophoresis patterns for C'3 typing of (A) serum from the liver donor, (B) serum from the liver recipient prior to transplantation, (C) serum from the liver recipient 20 hours after transplantation, and (D) serum from the liver recipient 6 days after transplantation. Prior to transplantation, the liver recipient was C'3 FS<sub>0.6</sub> while the donor was C'3 SS. Twenty hours after transplantation, the recipient's serum shows mostly C'3 SS with traces of C'3 FS<sub>0.6</sub>, while at 6 days after only C'3 SS is seen.

based on incorporation of radioactive amino acids into C'3 in tissue culture, has suggested that the cell of synthesis is the macrophage (15). Macrophages isolated from peritoneal and lung exudates were most active in the synthesis of C'3.

If the reticuloendothelial cells of the liver, the Kupffer cells, were responsible for hepatic production of C'3, since such tissue clearly represents less than half the total reticuloendothelial system of macrophages of the body, one would expect a mixture of recipient and donor C'3 types following transplantation of the liver.

*Note added in proof:* After submission of this manuscript for publication, a paper by Azen and Smithies (16) appeared describing their independent finding of C'3 polymorphism by high-voltage starch gel electrophoresis. On exchanging serum samples, it was established that their C3<sup>1</sup>, C3<sup>2</sup>, and C3<sup>3</sup> correspond to C'3 F, C'3 S, and C'3 S<sub>0.6</sub>, respectively, in our nomenclature (5).

CHESTER A. ALPER  
A. MYRON JOHNSON

Blood Grouping Laboratory and  
Children's Hospital Medical Center,  
Boston, Massachusetts 02115

ALAN G. BIRTCH  
FRANCIS D. MOORE

Peter Bent Brigham Hospital,  
Boston, Massachusetts 02115

#### References and Notes

1. D. A. Merrill, C. H. Kirkpatrick, W. E. C. Wilson, C. M. Riley, *Proc. Soc. Exp. Biol. Med.* **116**, 748 (1964).
2. N. Kashiwagi, C. G. Groth, T. E. Starzl, *ibid.* **128**, 247 (1968).
3. C. A. Alper, J. H. Peters, A. G. Birtch, F. H. Gardner, *Clin. Res.* **12**, 219 (1964); S. Krauss and E. J. Sarcione, *Biochim. Biophys. Acta* **90**, 301 (1964); H. Mouray, J. Moretti, M. Jayle, *Compt. Rend. Seances Acad. Sci.* **259**, 2721 (1964); C. A. Alper, J. H. Peters, A. G. Birtch, F. H. Gardner, *J. Clin. Invest.* **44**, 574 (1965).
4. J. H. Peters and C. Alper, *J. Clin. Invest.* **45**, 314 (1966).
5. C. A. Alper and R. P. Propp, *ibid.* **47**, 2181 (1968).
6. O. Smithies, *Biochem. J.* **71**, 585 (1959).
7. M. D. Poulik, *Nature* **180**, 1477 (1957).
8. C.-B. Laurell, *Anal. Biochem.* **15**, 45 (1966).
9. A. A. Boyden, E. T. Bolton, D. G. Gemeroy, *J. Immunol.* **57**, 211 (1947).
10. U. R. Nilsson and H. J. Müller-Eberhard, *J. Exp. Med.* **122**, 277 (1965).
11. P. Grabar and C. A. Williams, Jr., *Biochim. Biophys. Acta* **17**, 67 (1955); J. J. Scheidegger, *Int. Arch. Allergy Appl. Immunol.* **7**, 103 (1955).
12. C.-B. Laurell, *Anal. Biochem.* **10**, 358 (1965).
13. R. P. Propp and C. A. Alper, *Science* **162**, 672 (1968); C. A. Alper and R. P. Propp, in preparation.
14. C. A. Alper, A. S. Levin, F. S. Rosen, *J. Clin. Invest.* **44**, 1025 (1965); *Science* **153**, 180 (1966); C. A. Alper and F. S. Rosen, *J. Clin. Invest.* **46**, 2021 (1967).
15. G. M. Hochwald, G. J. Thorbecke, R. Asofsky, *J. Exp. Med.* **114**, 459 (1961); R. Asofsky and G. J. Thorbecke, *ibid.*, p. 471; G. J. Thorbecke, G. M. Hochwald, R. Van Furth, H. J. Müller-Eberhard, E. B. Jacobson, *Ciba*

- Foundation Symposium: Complement*, G. E. W. Wolstenholme and J. Knight, Eds. (Churchill, London, 1965), p. 99.
16. E. A. Azen and O. Smithies, *Science* **162**, 905 (1968).
  17. Supported in part by grants from the National Institutes of Health and the John A. Hartford Foundation, Inc. and by contracts with the Atomic Energy Commission and the United

States Army Medical Research and Development Command. We gratefully acknowledge the technical assistance of Miss Lillian Watson. We thank Dr. Louis K. Diamond for his critical reading of the manuscript. A.M.J. is the recipient of special fellowship 1 F3 AM-39,593 from the PHS.

20 September 1968

## Brown Adipose Cells: Spontaneous Mobilization of Endogenously Synthesized Lipid

**Abstract.** *Isolated brown adipose cells, devoid of a basement membrane, readily synthesized a variety of lipids from radioactive acetate, a reaction augmented by glucose and insulin. A large proportion of the newly formed fatty acids passed into the incubation medium. In intact brown adipose slices and isolated white adipose cells, most of the synthesized lipid was retained as glyceride esters. The data suggest that the rapid turnover of endogenously synthesized lipid in brown adipose cells is almost totally obscured in studies with intact tissue slices because of interstitial barriers to the egress of fatty acid.*

Conflicting views exist on the lipogenic activity of brown adipose tissue. It has been suggested that the low lipid content in brown fat may be the result of inadequate fatty acid synthesis (1), whereas other data have shown that lipogenesis in brown fat in vivo is active, and at times is equal to or greater than that in white fat (2, 3). My experiments show that lipogenesis in isolated brown adipose cells was brisk and sensitive to added glucose and insulin, and that a sizable proportion of newly synthesized fatty acid is spontaneously and preferentially released into the incubation medium. In white adipose cells retention is favored.

Male Wistar rats (180 to 200 g) raised on Purina chow were killed by a blow to the head, and the dorsal interscapular fat pad was excised. The pads were examined through a dissecting microscope, stripped of adherent white fat and interdigitating muscle, and cut into 5- to 10-mg pieces. The tissue fragments were added to a 30-ml plastic bottle containing 10 ml of Krebs-Ringer bicarbonate buffer with one-half the recommended concentration of Ca ion and 5 percent dialyzed bovine albumin. The buffer-albumin mixture had been frozen and was gassed to pH 7.4 with a mixture of O<sub>2</sub> and CO<sub>2</sub> (19:1) before use. Glucose was present at a concentration of 3 mmole/liter, and 10 mg of collagenase (4) was added.

The flask was capped and secured in a water bath (37°C), and the mixture was stirred with a small plastic-coated magnetic bar. Incubation continued until the next pad was ready to be sectioned; for each experiment three to

six pads were added this way. If the period of tissue handling exceeded 10 minutes, the pad was discarded. Treatment with collagenase continued for 45 minutes after the last addition of tissue. The incubation mixture was then filtered through surgical gauze moistened with fresh buffer-albumin solution to trap undigested tissue fragments and stromal and vascular elements. The filtrate was centrifuged at 300g for 1 minute, and the floating cells were transferred to another vial containing a known volume of buffer-albumin solution. Substrates were added, and the mixture was distributed in 1-ml volumes to plastic vials for final incubation. Methods for lipid extraction and measurement, radioassay, and thin layer chromatography have been described (5, 6).

Electron microscopy of the preparation of isolated cells demonstrated structural integrity and preservation of morphological features. Like isolated white adipose cells (6), brown cells were not enveloped by a basement membrane.

The conversion of acetate to lipid in isolated brown adipose cells was brisk, and, in the presence of 16 mM glucose, it was linear for at least 120 minutes at a velocity of 411 ± 81 nmole per millimole of cell fatty acid per hour (mean ± S.E.M., n = 5). The composition of lipids synthesized by isolated cells differed significantly from that produced by slices of brown adipose tissue (Table 1). In isolated cells, nearly half the newly formed lipid was in free fatty acid, whereas in intact tissue less than 2 percent was in free fatty acids, and over 90 percent of the lipid