ALTERATION IN BIOLOGICAL PROPERTIES OF HUMAN ALBUMIN DURING STORAGE

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SUMMARY: The effects of human albumin preparations on oxidative energy metabolism and lipid synthesis were investigated in rat liver slices incubated with sodium [1-^{14}C]acetate as precursor. Labeled CO_2 production and incorporation of precursor into the major lipid classes was increased 2 to 3-fold by fresh preparations of albumin (fraction V), and by defatted fraction V, whereas highly purified cystalline albumin was less active. Albumin preparations from various commercial suppliers varied widely in activity. Activity of fraction V was preserved during storage at -20°C, and gradually lost at +3°C in the course of 1 year. In contrast, defatted fractions rapidly lost activity in storage at both temperatures. After 1 year in storage at +3°C, albumin preparations became inhibitory to CO_2 production and lipid synthesis. The results suggest that commercial albumin used in metabolic studies, and in clinical situations may have unpredictable or undesirable effects related to state of purity and storage conditions of the protein.

The major plasma protein, albumin, is often considered to possess clearly defined biological properties within a given set of conditions in vivo and in vitro. However, many studies with cultured cells often refer to a poorly understood variability in the effects of various commercial albumin preparations on cell growth, intermediary metabolism, cellular transport functions and the binding of ligands (1-7). Different batches of either commercial Cohn fraction V albumin or crystalline albumin used in the same set of experiments, may be stimulatory, may have no effect, or may inhibit cell replication (1-5). These poorly understood differences in biological properties of human albumin may also have pertinence in clinical use of commercial albumin preparations. Patients with protein loss due to renal and intestinal disorders, extensive burns and surface infections often receive replacement therapy with intravenous infusions of commercial albumin. Hypoalbuminemia due to advanced liver disease may also require repeated albumin infusions. Newborn infants with severe hyperbilirubinemia routinely receive priming doses of albumin prior to

exchange blood transfusion (8). The administration of albumin with variable and occasionally inhibitory effects may have unpredictable and potentially undesirable effects in such patients.

In previous studies on the action of human and bovine albumin on liver intermediary metabolism (9), we noted a wide variation in the ability of various preparations of albumin to stimulate lipid synthesis and CO_2 production from energy substrates such as glucose, palmitate, acetate, and glutamic acid. More detailed studies of these variable effects of albumin preparations on hepatic lipid synthesis and CO_2 production from labeled acetate have led us to conclude that during storage albumin undergoes extensive changes which alter the biological properties of the protein.

METHODS:

Source of supplies. Human serum albumins (Cohn fraction V) and crystalline albumins were obtained from Cutter Laboratories, Berkeley, CA; Calbiochem, Los Angeles, CA; Nutritional Biochemicals, Cleveland, Ohio; Grand Island Biologicals, Berkeley, CA; Sigma Chemical Company, St. Louis, MO; Schwartz-Mann, Van Nuys, CA; Miles Laboratories, Kankakee, Illinois. Thixotropic gel and hyamine hydroxide (1M in methanol) were from the Packard Instrument Company, Burlingame, CA, thin layer chromatography plates and indicator sprays from Brinkman Instruments, Burlingame, CA, and phosphated buffered saline from Grand Island Biologicals. Sodium [1-^{14}C]acetate, 2mc/mM, was from New England Nuclear, Boston, MA.

Preparation of defatted albumin. Since albumin normally contains a variety of contaminants such as citrate, acetate, and free fatty acids, the most active albumin fraction (Cohn fraction V) was purified further and defatted by the trichloroacetic acid-ethanol-diether ether method described previously (11). This procedure reduced the molar ratio of free fatty acids to albumin from 1.9 in commercial Cohn fraction V to 0.14 in the final albumin preparation (11).

Studies with liver slices. Sprague-Dawley rats (200g) maintained on standard laboratory chow, were the source of liver used in all incubations. Animals were killed by decapitation, the liver removed, blotted dry, and sectioned on an automatic tissue sectioner set to produce 1 mm thick slices. The slices were weighed, and 100 mg portions were suspended in 2 ml of ice-cold serum containing 2μCi sodium [1-^{14}C] acetate and the albumin preparation to be tested. The time from sacrifice of the animal until incubation of slices was held to 20 minutes. The incubating serum was obtained from rats less than 1 hour before use. Human serum was substituted for rat serum in several experiments without changing the essential results.

Addition of albumin fractions up to 15 mg/ml to the incubating serum lowered the pH by less than 0.07 units. This decrease in pH had no effect on CO_2 production and lipid synthesis when reproduced with 0.15N HCL.

Incubation was in wide-bottomed 10 ml Erlenmeyer flasks filled with 100% oxygen, and attached tightly with gum rubber tubing to inverted U-shaped glass tops containing 0.3 ml hyamine hydroxide in an atmosphere of pure oxygen. The flasks were incubated at 37°C for intervals from 1 to 4 hours at a rotating speed of 100 rpm. Phenol red indicator was carried in test runs to ensure that the hyamine hydroxide was not accumulating in the incubation mix. Direct injection of hyamine hydroxide into the serum prior to incubation in quantities sufficient to produce a color change due to a pH shift, did not affect the results.

After incubation the flasks were packed in ice, and 1 ml of phosphate buffered saline (5X) at pH 5.0 was injected with a 27 gauge needle through the rubber tubing into the flasks. The sealed flasks were agitated for an additional 30 minutes to ensure complete release of $^{14}CO_2$ from tissues, the glass tops were detached, and the hyamine hydroxide containing the dissolved $^{14}CO_2$ transferred to counting vials. The radioactivity was then determined directly by counting in a standard toluene-based scintillation system.

Lipids were extracted from incubated tissue and serum using a modified Folch procedure (12). The lipid extracts were backwashed three times with 1/4 volume of an aqueous solution containing 10^{-3}M unlabeled acetate. Some of the extracts were chromatographed on thin layer silica-coated chromatography plates, in a system containing petroleum ether/diethyl ether/acetic acid (85:15:1). Lipid bands were located with exposure to iodine vapor and the iodine removed by sublimation. The lipid bands were scraped off into scintillation vials, and suspended in a thixotropic gel-scintillator slurry for radioactive counting.

Calculation of statistics. The basal production of carbon dioxide and lipid synthesis in the liver slices incubated in serum varied among experiments. For example, the incubation of 100 mg liver tissue for 2 hours with 2μCi of sodium [1-^{14}C]acetate yielded basal count rates of 9,000 to 20,000 cpm in carbon dioxide. This variability appeared to be primarily a function of the liver tissue as demonstrated by incubating slices from different animals in the same serum. Therefore, in each experiment the mean of the control values (e.g., liver slices plus serum) of radioactivity in carbon dioxide or lipids was considered as 100% and the effects of various protein fractions were expressed as a percentage of the control value for the calculation of variance. When using duplicate controls, the average deviation of counts in each sample from the mean was 7.3% for $^{14}CO_2$ production and 4.8% for lipid synthesis.

RESULTS AND DISCUSSION:

Effects of albumin fractions on CO_2 production and lipid synthesis in liver slices. Freshly prepared fraction V (albumin) was highly active in stimulating $^{14}CO_2$ production and lipid synthesis by liver tissue from labeled acetate (Tables I & II). Defatting of fraction V albumin by the trichloroacetic acid-ethanol-diethyl ether method, a procedure which removes bound lipids, caused negligible loss of activity. Highly purified crystalline albumin stimulated oxidation of acetate relatively weakly, albeit significantly, in comparison with the less pure albumin preparations. The albumin stimula-

TABLE I

Effect of human albumin preparations on the oxidation of [1-^{14}C]acetate by liver slices

Source	Albumin type added to serum*	No. of expts	$^{14}CO_2$ production % of control (control=100)	P value
Cutter Labs	Cutter's Cohn V	6	280	0.01
"	Repurified Cutter's V			
	@20 mg/ml	4	264	
	@15 mg/ml	14	245	0.001
	@10 mg/ml	4	221	
	@ 5 mg/ml	4	170	
Schwartz-Mann	Cohn V	6	125	
Sigma	Cohn V	6	118	
Nutritional Biochem	Cohn V	6	190	
Miles	Cohn V	6	138	
Grand Island Biol	Cohn V	6	137	
Calbiochem	Crystalline	6	120	
Sigma	Crystalline	6	106	
Schwartz-Mann	Crystalline	6	117	

*All proteins at 15 mg/ml except where noted.

TABLE 2

Albumin stimulation of acetate oxidation and incorporation into lipid classes by rat liver slices.

Metabolite measured	Control cpm	Plus albumin cpm
CO_2	3800	11400
Phospholipids	2550	8990
Cholesterol	4370	9030
Free fatty acids	5620	9070
Triglyceride	3790	9190
Cholesterol Esters	2300	2770

Each incubation flask contained 2 ml serum from fed rats, 100 mg liver slices from fed rats, and 2 microcuries (1-^{14}C)acetate. Incubation was for 2 hours. Added albumin (repurified human Cohn fraction V) was 15 mg/ml. Each value represents the average of three flasks.

tion of lipid synthesis affected all lipid classes with the exception of cholesterol esters (Table II). Phospholipid synthesis was stimulated most effectively.

The most active albumin preparations (Cohn fraction V) were from Cutter Laboratories, obtained 1 day to 2 weeks after preparation from human plasma, transported on ice, and stored at -20°C before use. Human albumin, both as fraction V and in crystalline form, from other suppliers (Sigma, Schwartz-Mann, Calbiochem, Miles, Nutritional Biochemicals) contained from 6% to 38% of the activity in fraction V from Cutter Laboratories.

The possibility existed that the stimulatory effect of freshly prepared albumin on liver slices was due to the maintenance of a suboptimal metabolic

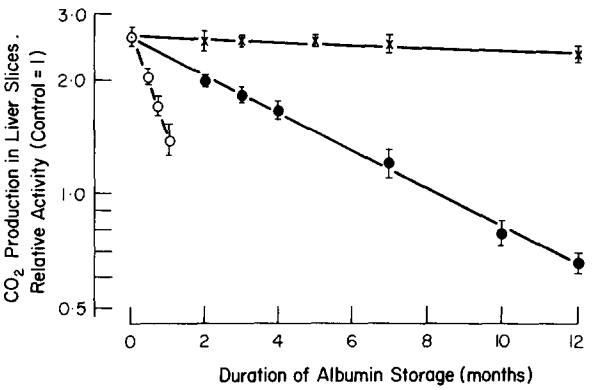

Fig. 1 Changes in stimulatory effects of human albumin during storage. Relative activity represents the ratio of $^{14}CO_2$ produced by liver slices (100 mg) incubated with added albumin (15 mg/ml) and without added albumin. Incubations were in 2 ml serum for 2 hours at 37°C. Each point represents the mean of 6 experiments ± S.E.M.
(-X-) represents fraction V stored at -20°C
(-●-) represents fraction V stored at +3°C
(-0-) represents defatted fraction V stored at -20°C

system, analogous to effects reported in preparations of metochondria treated with albumin (13,14). An analysis of the results, however, suggests that this is not the case. Lipid synthesis from acetate is a sensitive index of metabolic integrity in liver cells. The ability to utilize acetate for lipid synthesis is lost in isolated hepatocytes which are partially damaged, whereas near normal rates of protein synthesis are maintained (15). Similarly, the *in vitro* conversion of acetate into CO_2 and lipids by liver cells is completely blocked by inhibitors of protein synthesis at concentrations which have no effect on the rate of conversion of [2-^{14}C]mevalonic acid into CO_2 and cholesterol (16). Therefore, it would seem likely that the effect of albumin is a physiologically meaningful result and not an artifact due to tissue damage. In addition, when albumin preparations (fraction V, re-purified and defatted and time-inactivated albumin) were tested in intact mice, their effects on $^{14}CO_2$ production *in vivo* from [1-^{14}C]acetate corresponded to their effects in liver slices (9).

Effects of storage conditions on albumin activity. Fraction V retained its stimulatory activity when stored at -20°C, whereas activity gradually disappeared during a 6-month period of storage at 3°C (Fig. 1). The loss in activity appeared to follow first order kinetics during 6 months of storage at 3°C. Defatted fraction V lost its stimulatory activity rapidly even when stored at -20°C. Albumin (human fraction V) samples stored for more than 1 year in this manner acquired an inhibitory activity with regard to CO_2 production (Fig. 1).

The decrease in the stimulation of CO_2 production from acetate was paralleled by a similar decrease in lipid synthesis. All albumin samples which inhibited CO_2 production from acetate also inhibited lipid synthesis.

Losses of activity from albumin stored at relatively warm temperatures or from defatted albumin suggest that a process of oxidation may be responsible for the changes observed during storage. According to an informal survey of commercial outlets handling albumin, most pharmaceutical and manufacturing supply houses store their albumin stocks at 2°C to 4°C. Our results suggest that under these storage conditions, the "metabolic" activities of albumin become degraded with time. Thus, storage of albumin may be a factor in the development of variability and microheterogeneity observed with commercial albumin preparations.

Two mechanisms have been proposed for the appearance of microheterogeneities observed after storage of purified albumin fractions. Tappel proposed that metal ions such as copper, which are bound to albumin, catalyze the oxidation of unsaturated fatty acids to derivatives which then react with the ε- amino groups of lysine to form irreversible complexes. Highly purified and crystallized albumins give more evidence of such oxidation products than less pure albumin preparations such as Cohn fraction V (17). Crystallized albumin had low or negligible stimulatory activity in our experiments. An alternate explanation of albumin microheterogeneity advanced by Wallerick, is that an irreversible oxygen-catalyzed interchange of disulfide bridges occurs in the

protein during storage which leads to the development of at least five forms of albumin with similar isoelectric points (18).

In view of extensive clinical use of albumin, these results and the cited work on occasional inhibitory effects of the protein in cell culture (1-5), suggest that the effects of various methods of preparation and storage of human albumin deserve further study.

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