Since p-CF_3O-CCP was reported to be highly active as an uncoupling agent in mouse liver mitochondrial preparations(1), a further attempt to observe stimulatory effects of p-CF_3O-CCP in intact mice was made. The dose was increased from 11 mg/kg to 15 mg/kg but this slightly higher dose of p-CF_3O-CCP killed enough mice so as to prevent measurements of MR. Further experimentation is necessary in order to determine the reason for lack of demonstrating increased MR with p-CF_3O-CCP in the intact mouse. Furthermore, since higher doses of p-CF_3O-CCP killed mice without gross indications of increased MR, the primary toxic effect responsible for death of mice may not be associated with uncoupling of phosphorylation *per se*.

Summary. In rats, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (p-CF_3O-CCP) was more potent than 2,4-dinitrophenol (DNP) in causing marked oxidative and expiratory effects but was without apparent effect in mice at an equivalent dose. Toxicity of p-CF_3O-CCP in mice prevented further observations since increasing the dose proved lethal. DNP was active in both mice and rats.

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Studies on Soluble Antigenic Substances Isolated from Human Liver. (28394)

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Tissue homogenates, as well as specific cellular components from a variety of mammalian organs, are reactive in complement fixation (CF) tests with serum substances from patients with systemic lupus erythematosus (S.L.E.) and other diseases(2-10). These reactive tissue components include cellular nuclei, mitochondria, microsomes, and soluble cytoplasmic factors isolated from human, rat, rabbit, and calf liver(2,6-8), as well as fractions from calf thymus(5-7), human kidney(7), and thyroid(9) and human and rabbit pancreas(10). The reactivity of cellular nuclei and deoxyribonucleic acid-protein complexes in CF tests has been well documented with sera from persons with S.L.E.(5-7,11). In addition, substances in the cytoplasm of cells have also been shown to react with sera from patients with S.L.E. (2,6-8). These cytoplasmic substances, however, have not been adequately characterized. This report concerns their isolation, fractionation, and chemical characterization.

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Materials and methods. Human liver obtained at autopsy from young accident victims 6-12 hours post mortem was frozen and stored at –60°C. For antigenic isolation, each gram of liver was extracted for one minute in a Virtis homogenizer with either 9 ml of 0.15 M sodium chloride or 0.44 M sucrose. All processes were conducted at 1° to 3°C unless otherwise indicated. The cell nuclei and tissue debris were removed by immediate centrifugation (10 min. at 700 × *g*) of the homogenate. The sediment was washed once with the extractant and the wash combined with the supernatant solution. Sucrose was the extractant of choice when cellular constituents were desired, and these were separated by differential centrifugation(7). For isolation of the soluble fraction, the clear supernatant solution remaining after centrifugation for 1 hr at 105,000 × *g*, 0.15 M sodium chloride was used.

The soluble fraction was further fraction-

ated by precipitation under controlled conditions of pH and ionic strength. The solution was adjusted to pH 7.4 with 0.01 N sodium hydroxide and 3 volumes of 24% ethanol added. The precipitation was allowed to proceed for at least 6 hours at −5°C. The precipitate was recovered by centrifugation (30 min at 2000 × *g*) and washed once with a solution containing 1 part of 0.15 M veronal buffered saline† (VBS) and 3 parts 24% ethanol. The washed precipitate was dissolved in 0.15 M VBS (5 ml per gram liver giving a 1:5 dilution), and designated fraction I. The wash was combined with the supernatant solution.

The solution was then adjusted to pH 6.4 with 0.1 N acetic acid, and processed as before, except that the wash solution was 0.025 M acetate buffer pH 6.4 containing 16% ethanol. This precipitate was designated as fraction II. The procedure was repeated with the solution at pH 5.9 and 5.2, and the recovered precipitates designated fractions III and IV. The wash solutions for fractions III and IV were 0.025 M acetate buffer (pH 5.9 and 5.2) containing 16% ethanol. The solution which remained after removing fraction IV was serologically inactive in CF tests with the sera studied and was discarded. The fractions were compared in CF tests using a constant amount of antibody.

The serologically active fractions I, II, and IV (III was non-reactive) were subjected to the action of trypsin and ribonuclease (RNAse).‡ Any changes in serological activity of the fractions were then ascertained in CF tests. One mg of trypsin was added to 1 ml of the soluble fraction (1:5 dilution) and permitted to react for 1 hr at 37°C. Then trypsin inhibitor, equal to the amounts of trypsin, was added to stop the reaction. Finally, an aliquot of the trypsin digest was treated further with 1 mg RNAse for 1 hr. This sequence of steps was adopted because fractions I and II became anticomplementary when heated at 37°C for 1 hr. Prior treatment with trypsin prevented this anticomplementary effect, and permitted measurement of the action of RNAse.

The possibility that the active serological substances were extracted from the cell nuclei was considered with two different approaches: 1) the liver tissue was extracted for varying lengths of time and the extracts compared in serological tests, 2) nuclei were separated from other cell constituents by a non-aqueous gradient centrifugation method (12). The isolated nuclei were extracted with 0.15 M sodium chloride as described for tissue.

The sera used were from patients whose diagnosis of S.L.E. was substantiated by both clinical and laboratory findings. The CF test employed was a precisely standardized method(13).

Results. Sodium chloride (0.15 M) and sucrose (0.44 M) were equally effective in extracting serologically active substances found in the soluble fraction. Previous investigations had shown, however, that appreciable amounts of nuclear material are soluble in 0.15 M sodium chloride(14). Therefore, a study was undertaken to determine if the serologically active substances in the soluble fraction were derived from the nuclei. Serological reactivities of a 0.15 M sodium chloride extract of the whole liver tissue, treated for varying lengths of time, the nuclei, isolated by non-aqueous differential centrifugation(12) and the non-nuclear portion of the non-aqueous extract are given in Table I.

The entire aqueous extraction process, including centrifugation for 10 minutes, allowed contact of the nuclei with the 0.15 M sodium chloride for a maximum of 15 minutes. Lengthening the extraction time to 6 hours produced no increase in serological activity. The 15 minute saline extract of nuclei isolated in a non-aqueous system contained no detectable CF antigenic substances with the studied sera. If the extraction was allowed to continue for 24 hours, however, some serologically active material was obtained, possibly from the extensive rupture

† Prepared by dissolving 2.3 g 5,5′ diethylbarbituric acid in 500 ml hot distilled water, then adding 1.5 g sodium 5,5′ diethylbarbiturate, 1.26 g sodium bicarbonate, and 41.90 g sodium chloride. Solution was cooled and diluted to 1 liter. This stock solution, when diluted 1:5 with distilled water, had a pH 7.3-7.4.

‡ Purchased from Worthington Biochemical Corp., Freehold, N. J.

TABLE I. Complement Fixing Reactivity of Saline Extracts of Human Liver with Systemic Lupus Erythematosus Serum.

Fraction and extraction time	Fraction diluted 1: 10	20	40	80	160	Serum control
Soluble fraction						
1. ¼ hr	0*	0	0	30	—	—
2. 6 "	0	0	0	25	—	—
Nuclei (non-aqueous)						
1. ¼ hr	—	—	—	—	—	—
2. 24 hr	0	5	40	—	—	—
Non-nuclear (non-aqueous)						
1. ¼ hr	0	0	0	25	—	—

* Numerical values represent percent hemolysis, negative sign (—) indicates complete hemolysis.

of nuclei. A saline extraction of the non-nucleic portion of the non-aqueous extract for 15 minutes, on the other hand, produced the usual amount of serologically active substances. The results suggest, therefore, that the serologically active substances found in the soluble fraction were not derived from the nuclei.

The stepwise precipitation of the soluble fraction resulted in the isolation of serologically distinct antigenic substances (Table II). The fractions I, pH 7.4, II, pH 6.4, and IV, pH 5.2 all reacted with sera from the patients with S.L.E. but to a different degree. All 3 reacted with the first S.L.E. serum, but IV reacted most strongly. I and II, on the other hand, reacted strongly with the second L.E. serum while IV was considerably less reactive. Only I and II reacted with the serum from the patient with thrombocytopenic purpura, and in this case, II was the more reactive.

It has been reported(2,6) and also observed in our laboratory, that sera from certain syphilitic patients reacted with tissue extracts. In addition, it has also been observed in our laboratory that human liver tissue extracts reacted in CF tests with sera from healthy rabbits. The data in Table II show that fraction I reacted most strongly with the luetic serum, while fraction II reacted most strongly with the normal rabbit serum. The fractions were not anticomplementary as shown by the antigen control. It should also be noted that none of these fractions reacted with sera from 25 healthy persons, 21 containing rheumatoid factor, nor with the sera from 12 normal rats and 16 guinea pigs. These results would indicate a multiplicity of substances in the soluble fraction from human liver which reacted in CF tests with sera from persons with S.L.E. and other studied diseases. The results also suggest that the serum substances (antibodies) which reacted with different fractions need not be the same in persons with the same clinical diagnosis.

The serologically reactive fractions (I, II, IV) were investigated with trypsin and RNAse. The fractions were treated first with

TABLE II. Complement Fixing Reactivity of Substances Isolated from Soluble Fraction of Human Liver.

Fraction	dil'd 1:	% hemolysis with studied sera S.L.E.	S.L.E.	Th.P.	Luetic	NRS	Antigen control
	5	0*	0	5	10	60	—
I	10	0	0	25	20	80	—
	20	70	0	—	80	—	—
	40	—	30	—	—	—	—
	5	0	0	0	70	0	—
II	10	10	0	10	—	50	—
	20	70	0	80	—	80	—
	40	—	20	—	—	—	—
	5	0	20	—	—	90	—
	10	0	50	—	—	—	—
IV	20	0	70	—	—	—	—
	40	0	—	—	—	—	—
	80	20	—	—	—	—	—

Th.P.—Thrombocytopenic purpura, NRS—Normal rabbit serum.
* Numerical values represent percent hemolysis, negative sign (—), indicates complete hemolysis.

TABLE III. Effects of Trypsin and Ribonuclease on Complement Fixing Reactivity of Substances Isolated from Soluble Fraction of Human Liver.

		S.L.E. sera					
		I			II		
Fraction	dil'd 1:	Sal.	Try.	RNAse	Sal.	Try.	RNAse
I	5	0*	0	50	0	40	90
	10	0	70	—	0	50	—
	20	70	—	—	0	60	—
	40	—	—	—	30	70	—
II	5	0	0	0	0	15	0
	10	10	20	50	0	20	20
	20	70	—	—	0	40	70
	40	—	—	—	20	70	—
IV	5	0	0	0	20	70	—
	10	0	0	0	50	80	—
	20	0	0	30	70	—	—
	40	0	0	—	—	—	—
	80	20	30	—	—	—	—

* Numerical values represent percent hemolysis, negative sign (—) indicates complete hemolysis.

trypsin, the reaction stopped with inhibitor, and then an aliquot treated with RNAse. Controls containing no enzymes were tested concurrently, but were not heated at 37°C. Results obtained with the enzyme treated fractions and controls using 2 sera from patients with L.E. are given in Table III.

The 3 fractions, studied in CF tests with the sera from persons with L.E., responded differently after treatment with the enzymes. Serological activity of fraction I was decreased with both sera after the trypsin digest, and decreased still more by the action of RNAse. Fraction II, on the other hand, was affected little, if any, by the action of either trypsin or RNAse. The apparent small changes appeared to be of little significance. Fraction IV, when reacted with L.E. serum I, was not affected by trypsin, but the antigenic activity was decreased significantly by RNAse. The reaction of fraction IV with S.L.E. serum II was so weak that the results were difficult to interpret. While not large, the decreased antigenic activity caused by the enzymes was reproducible. These results suggested that the substances which acted as antigens in CF tests with sera from persons with L.E. might be RNA-protein and also possibly protein. They also suggest that the antibody specificity of S.L.E. sera I and II differed.

Discussion. Serologically active substances extracted from human liver were solubilized equally well by either 0.15 M sodium chloride or 0.44 M sucrose. When sodium chloride was used, the cellular particulate matter could not be fractionated by differential centrifugation. When sucrose was used, the soluble portion could not be further fractionated by isoelectric precipitation. The differences found with these solvents probably resulted from the change in protein-protein interaction in the unionized sucrose and the highly ionized sodium chloride. Under the experimental conditions, neither sodium chloride nor sucrose extracted serologically active substances from the isolated nuclei. The possibility, however, that the organic solvents changed the nuclear membrane permeability, has not been excluded.

The soluble fraction from human liver was previously extracted with sucrose and fractionated(1) by method 10 of Cohn *et al.* (15). The serological activity was found exclusively in fraction III-0. However, attempts to further purify this fraction resulted in considerable loss of antigenic activity in CF tests. On the other hand, the stepwise precipitation under controlled conditions of decreasing pH at low ionic strength resulted in the isolation of 3 serologically distinct fractions with no apparent loss of activity.

The loss of serological reactivity of two fractions, I and IV (Table III) after treatment with RNAse suggested that RNA-pro-

tein are reactive with serum substances from persons with S.L.E. The possibility also exists that proteins other than nucleo-proteins are involved in these reactions. On the other hand, the antigenic activity of mitochondria and microsomes was found by other investigators to be unaffected by the action of trypsin of RNAse(7).

The serological reactivity of the studied sera with the 3 fractions (Table II) emphasized the complexity of the antigen-antibody reactions in these systems. The presence of multiple antibodies in S.L.E. has been reported(7). The results of this study support and extend these results. The dissimilarity of reactivity of the 2 S.L.E. sera could not be correlated with any known data. These results may demonstrate dissimilarities in degree of antibody response to different antigens by individuals or possibly different antibody responses in various stages of the disease.

The cross reactivity between human tissue and rabbit sera was expected. Gajdusek(3) had previously found cross reactions between rat liver and kidney tissue and human sera. The cross reactivity of the tissue homogenates with luetic sera has also been reported by others(2,6-8). It was interesting that the luetic serum reacted strongly only with fraction I. Absorption studies have shown that Wasserman and Forssman antibodies are apparently not involved in these reactions(2,7, 8).

The antigenic activity of the soluble fraction from human liver was found recently to be associated with proteins which were similar to 7s-gamma globulin(16). It was suggested also that the observed complement fixing resulted not from antigen-antibody reactions but from aggregations of normal tissue and serum gamma globulin.

Summary. Non-particulate cellular cytoplasmic constituents from human liver were isolated by physicochemical procedures. These substances were reactive as antigens in complement fixation tests against sera from patients with lupus erythematosus and other diseases. Results indicated that the soluble fraction constituents were not extracted from the cell nuclei. Fractionation of the soluble fraction by isoelectric precipitation at low ionic strength produced 3 active precipitates each of which reacted differently in complement fixation tests with sera from patients with lupus erythematosus. Enzymatic studies suggested that the substance active as antigens were RNA-protein and protein.

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