hours after injection of hydrocortisone, exhibited more pronounced hypocalcemia than PTX controls. In non-PTX rats the serum Ca was not lowered after injection of the steroid. Comparing the effect of graded doses of bovine PTE (5-90 U.S.P. units) upon the serum Ca of PTX, hydrocortisone-injected rats and of PTX controls 3.5 times more PTE had to be administered to the hydrocortisone-injected group to sustain normal serum Ca concentrations in the 2 groups of PTX animals. Thus it appears that the parathyroids of hydrocortisone-injected rats secrete about 3 times more hormone than those of non-injected controls. The serum Ca-lowering effect of hydrocortisone was not a transient one and was demonstrable after 3 weeks of daily treatment. The effect of hydrocortisone upon the blood Ca of PTX rats was not mediated by circulating citrate.

1. Leifer, E., Hollander, W., *J. Clin. Endocrinol.* 1953, v13, 1264.

2. Mirvish, L., Bosman, L. P., *Brit. J. Exp. Biol.*, 1929, v6, 350.

3. Stoerk, H. C., Arison, R. N., *Inflammation and Diseases of Connective Tissue,* A Hahnemann Symposium, Lewis C. Mills and John H. Moyer, Ed., W. B. Saunders Co., Philadelphia, 1961, p399.

4. Berger, E. Y., *Clin. Chem.*, 1955, v249.

5. Beutler, E., Yeh, M. K. Y., *J. Lab. Clin. Med.*, 1959, v54, 125.

6. Harrison, H. C., Harrison, H. E., Park, E. A., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 768.

7. Agrell, I. G., Lindell, S. E., Westling, H., *Acta Med. Scand.*, 1961, v169, 127.

8. Freeman, Smith, *The Parathyroids,* R. O. Greep, R. V. Talmage, Ed., Charles C Thomas, Springfield, Ill., 1961, p262.

9. Unpublished observations.

10. Harrison, H. E., Harrison, H. C., *Am. J. Physiol.*, 1960, v199, 265.

11. Stoerk, H. C., Boeninghaus, J., Celozzi, E., *Fed. Proc.*, 1963, v22.

12. Nordin, B. E. C., *Lancet,* 1961, v1, 1011.

Received September 3, 1963. P.S.E.B.M., 1963, v114.

## Effect of Endotoxin-Tolerance, Cortisone, and Thorotrast on Release of Enzymes from Subcellular Particles of Mouse Liver.* (28773)

AARON JANOFF AND B. W. ZWEIFACH

*Department of Pathology, New York University School of Medicine, New York City*

*In vitro* studies by Weissmann(1,2) on a large-granule fraction of rabbit liver homogenate have shown that release of acid-hydrolases from the lysosomes of this fraction can be partially inhibited by pretreatment of the rabbits with cortisone or by prior adaptation of the animals to bacterial endotoxin. In our laboratory, adaptation of rats to traumatic shock (drum-tolerance) was also found to inhibit the release of acid-hydrolases from hepatic lysosomes when the corresponding granule fraction was exposed to ultraviolet irradiation *in vitro*(3). In view of deDuve's suggestion that the acid-hydrolases contained within these particles may contribute to the destruction of cells during pathological states(4), we and others have postulated that stabilization of lysosomal membranes in tolerant animals might constitute a mechanism of adaptation to trauma and toxic substances(1,3). As a further extension of these studies in adapted animals, experiments have been undertaken to determine whether stabilization of lysosomal membranes reflects a general alteration in permeability of subcellular particles resulting from tolerance, or represents instead a special response of lysosomes to repeated stress. In addition, since tolerance to endotoxin or trauma can be overcome by injection of a reticuloendothelial-blocking agent such as Thorotrast(5), we also investigated the effect of administration of this agent upon the *in vitro* release of acid-hydrolases from lysosomes of normal and endotoxin-tolerant mice.

* Supported in part by grants from Office of the Surgeon General, Dept. of the Army and Nat. Inst. Health (HE-07289).

*Methods and materials.* Male, Swiss-Webster mice weighing 25 to 30 g were used in all experiments. Tolerance to bacterial endotoxin was produced by intravenous injection of 17 gamma of *Salmonella enteritidis* endotoxin (Difco lot #127402) daily for 4 days. Endotoxin-treated mice were killed and their livers removed 24 hours after the last dose of the lipopolysaccharide. Cortisone (Cortone-acetate, Merck & Co.) was injected intraperitoneally in doses of 3.7 mg per mouse. One hour later, animals were sacrificed and livers removed. In one experiment, colloidal thorium dioxide (Thorotrast, Testagar Co.) was injected intravenously into normal and endotoxin-tolerant mice in doses of 25 mg per animal. Livers were removed from these mice 2 hours after the injection of colloid.

In all experiments, pools of 3 livers from similarly treated animals were used in preparation of individual particulate fractions. Each pool of 3 livers was homogenized in ice-cold 0.25 M sucrose and a large-granule fraction (LGF) was isolated by methods previously described(1). The washed LGF was resuspended in 0.25 M sucrose and divided into 3 equal aliquots. Two of these were diluted to a standardized, final volume with additional sucrose, and the third with sucrose which had previously been saturated with carbon tetrachloride (final concentration of carbon tetrachloride in this aliquot = $3.3 \times 10^{-3}$ M). One of the 2 sucrose aliquots was blendorized at high speed in the microcup of the Waring Blendor, then all 3 aliquots were incubated at 37°C for 40 minutes, after which they were centrifuged at 17,000 $\times$ *g* for 20 minutes to obtain clear supernates for assay of enzymes liberated during the incubation. Lysosomal beta-glucuronidase activity was measured by the method of Fishman(6), lysosomal acid-phosphatase by the method of Huggins and Talalay(7) with phenolphthalein diphosphate as substrate, mitochondrial malic dehydrogenase by Mehler's method(8), and mitochondrial isocitric acid dehydrogenase by the method of Bowers(9). Enzyme activity released by $CCl_4$ incubation was calculated as per cent of activity released in a corresponding aliquot which had been exposed to the Waring Blendor (the latter value represented total activity present in the sample). Carbon tetrachloride-induced lysis of lysosomes and mitochondria was selected in place of ultraviolet irradiation, when it was found that both malic- and isocitric acid-dehydrogenase were sensitive to UV light. Activity of none of the 4 enzymes was affected by carbon tetrachloride at the concentration employed.

*Results.* In the control mice, incubation of LGF of liver homogenates in the presence of $10^{-3}$ M $CCl_4$ accelerated the release of soluble enzymes from lysosomes and mitochondria, in comparison to the rate observed in samples incubated without this agent. Leakage of beta-glucuronidase and acid-phenolphthalein-phosphatase from lysosomes was usually doubled by $CCl_4$, while release of malic dehydrogenase from mitochondria was increased almost 3-fold (Table I). Leakage of isocitric acid dehydrogenase from control mitochondria incubating at 37°C reached levels as high as 50% of the total particle-bound activity of the sample, and addition of $CCl_4$ to the incubation mixture did not cause further release of this enzyme.

Table I also shows the effect of endotoxin-tolerance and cortisone-pretreatment on the rate of release of enzymes from mouse liver LGF. Appearance of lysosomal beta-glucuronidase in the supernate of particle suspensions incubating with $CCl_4$ was significantly less in both endotoxin-tolerant and cortisone-treated groups. Release of acid-phenolphthalein-phosphatase was also significantly reduced in the case of endotoxin-tolerant mice, but not in the cortisone-treated group. However, rate of leakage of soluble mitochondrial dehydrogenases was clearly not reduced by endotoxin-tolerance or cortisone-pretreatment.

Table II shows the results of a single experiment in which the effect of Thorotrast treatment on rate of leakage of the 2 acid-hydrolases and of malic dehydrogenase from liver particles of control and endotoxin-tolerant mice was studied. Reduction in release of beta-glucuronidase and acid-phosphatase was observed in the tolerant mice as before, but was much less pronounced in animals whose tolerance had been reversed by Thoro-

TABLE I. Effect of Endotoxin-Tolerance and Cortisone on Release of Enzymes* from Mouse Liver Particles.

G (beta glucuronidase); AP (acid phenolphthalein phosphatase); MDH (malic dehydrogenase); ICDH (isocitric acid dehydrogenase).

| Exp group | No. mice (No. liver pools) | Enzyme | Incubation alone (37°C × 40 min) | Incubation with $CCl_4$ ($3.3 \times 10^{-3}$M) |
|---|---|---|---|---|
| Control | 48 (16) | G | 20 ± 5 | 39 ± 5 |
| | | AP | 20 ± 4 | 50 ± 6 |
| | | MDH | 10 ± 3 | 27 ± 3 |
| | | ICDH | 52 ± 2 | — |
| Endotoxin tolerant | 30 (10) | G | 15 ± 5 | 24 ± 4‡ |
| | | AP | 12 ± 1† | 18 ± 1† |
| | | MDH | 16 ± 4 | 37 ± 7 |
| | | ICDH | 54 ± 5 | — |
| Cortisone treated | 30 (10) | G | 14 ± 2§ | 24 ± 3‡ |
| | | AP | 24 ± 2 | 55 ± 4 |
| | | MDH | 11 ± 1 | 36 ±10 |
| | | ICDH | 53 ± 8 | — |

* Values given in table represent avg (± S.D.) % of total activity released into supernate under incubation conditions shown.

Difference between control and experimental value is significant ("T" test) at: † 0.1% level. ‡ 0.5% level. § 2.5% level.

TABLE II. Effect of Thorotrast on Release of Enzymes from Mouse Liver Particles.

| Exp group | No. mice (No. liver pools) | Enzyme | Incubation alone | Incubation with $CCl_4$ |
|---|---|---|---|---|
| Control | 6 (2) | G | 13, 15 | 25, 26 |
| | | AP | 16, 19 | 26, 28 |
| | | MDH | 10, 10 | 34, 36 |
| Endotoxin tolerant | 6 (2) | G | 7, 9 | 18, 20 |
| | | AP | 4, 6 | 8, 9 |
| | | MDH | 12, 15 | 37, 37 |
| Endotoxin tolerant after Thorotrast | 6 (2) | G | 12, 13 | 20, 22 |
| | | AP | 10, 11 | 19, 21 |
| | | MDH | 9, 10 | 31, 33 |
| Control after Thorotrast | 6 (2) | G | 18, 18 | 35, 37 |
| | | AP | 14, 15 | 38, 39 |
| | | MDH | 9, 10 | 38, 40 |

trast-treatment. Moreover, release of acid-hydrolases from lysosomes of normal (non-tolerant) mice was increased after administration of Thorotrast. On the other hand, leakage of malic dehydrogenase from mitochondria was not increased in the Thorotrast-treated animals.

*Discussion.* The foregoing observations reveal that stabilization of hepatic lysosomal membranes occurs in the mouse as well as in the rabbit(1) after endotoxin tolerance. Moreover, the stabilization afforded to these particles by endotoxin-tolerance is clearly effective against $CCl_4$-induced lysis as well as against lysis following UV-irradiation(1). Although cortisone-treatment reduced the release of lysosomal beta-glucuronidase in our system, release of acid-phenolphthalein-phosphatase was not inhibited by this agent.

Neither endotoxin-tolerance nor cortisone-pretreatment stabilized mitochondrial membranes, in spite of the fact that lysosomes present in the same granule fraction were so affected. Some degree of selective stabilization of hepatic lysosomes induced by chronic sub-lethal endotoxinemia is thus apparent; at least this response is not shared by liver mitochondria of mice treated with this agent. In view of the biochemical evidence of early degenerative changes in liver lysosomes following administration of endotoxin(1) and the appearance of lysosomal hydrolases in the

blood stream after lethal doses of this toxin (3), the selective stabilization of hepatic lysosomal particles in endotoxin-tolerant animals would seem to represent an important component of tolerance to this agent. Such an interpretation is also favored by the observation that Thorotrast increased the fragility of liver lysosomes but did not similarly affect mitochondria. Thus, an agent which overcomes tolerant states exhibited a selective labilizing action upon lysosomal membranes in our test system.

*Summary.* Carbon tetrachloride-induced leakage of 2 acid-hydrolases from mouse liver lysosomes, *in vitro,* was inhibited by chronic pretreatment of the mice with endotoxin. Cortisone-pretreatment inhibited the release of one of the enzymes. Pretreatment of the mice with Thorotrast, a reticulo-endothelial-blocking agent which neutralizes tolerant states, had the opposite effect and caused accelerated release of acid-hydrolases from liver lysosomes of tolerant as well as normal animals. None of the 3 agents studied produced comparable changes in the rate of leakage of soluble dehydrogenases from mouse liver mitochondria. It is suggested that stabilization of hepatic lysosomal membranes in endotoxin-tolerant animals represents a selective response of these particles to repeated small doses of the toxin and plays an important role in the development of tolerance to this agent.

The technical assistance of Miss Sonja Schaefer and Mr. Martin Rosenberg is gratefully acknowledged.

1. Weissmann, G., Thomas, L., *J. Exp. Med.,* 1962, v116, 433.

2. ———, *J. Clin. Invest.,* 1963, v42, 661.

3. Janoff, A., Weissmann, G., Zweifach, B. W., Thomas, L., *J. Exp. Med.,* 1962, v116, 451.

4. de Duve, C., *Subcellular Particles* (T. Hayashi, ed.), 1959, N. Y., Ronald Press Co., 128.

5. Zweifach, B. W., Thomas, L., *Res. Bull.,* 1957, v3, 31.

6. Fishman, W. H., Springer, B., Brunetti, R., *J. Biol. Chem.,* 1948, v173, 449.

7. Huggins, C., Talalay, P., *ibid.,* 1945, v159, 399.

8. Mehler, A. H., Kornberg, A., Grisolia, S., Ochoa, S., *ibid.,* 1948, v174, 961.

9. Bowers, G. N., Jr., *Clin. Chem.,* 1959, v5, 509.

Received September 3, 1963. P.S.E.B.M., 1963, v114.

## Serum Haptoglobin Types in Rheumatoid Spondylitis.* (28774)

ALAN S. COHEN AND WILLIAM CHASEN

*Robert Dawson Evans Department of Clinical Research, Massachusetts Memorial Hospitals and Boston University School of Medicine, Boston University Medical Center, and the Arthritis Clinic, Veterans Administration Outpatient Clinic, Boston*

In the few years since Smithies separated by starch gel electrophoresis a number of hemoglobin binding proteins (haptoglobins), a large body of information has accumulated regarding the genetic control of these substances(1-4). Although the early studies indicated that 3 haptoglobin phenotypes (1-1, 2-1, and 2-2) were representative of 3 genotypes produced by a single pair of allelomorphic genes ($Hp^1$ and $Hp^2$) more recent studies have described other rarer phenotypes and techniques of subtyping have been introduced(5).

Since haptoglobins and other serum protein factors are inherited and since other blood substances (especially blood groups) have been associated with specific disease states, an analysis of the relation that might exist between haptoglobin types and rheumatoid spondylitis was carried out. The latter disease was chosen because there is good evidence that genetic mechanisms play a role in its genesis(6).

*Methods and materials.* Forty-five adult

* Grants in support of these investigations have been received from Nat. Inst. of Arthritis and Metab. Dis., and Mass. Chapter of the Arthritis and Rheumatism Foundation.