Abnormal Serum Protein Observed in Trypan Blue-Treated Rats.* (25017)

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Trypan blue has been known to bind with albumin and also with α-globulin when the quantity of dye is sufficient(1,2). It is also known that thyroxine binds with specific serum protein which has an electrophoretic mobility between that of α-1 and α-2 globulin at pH 8.6(3,4). Recently Crispell *et al.*(5) reported an *in vitro* experiment indicating that trypan blue competes with thyroxine for binding sites on the "thyroxine-binding protein," which thus would make more thyroxine available to the cells because of the weaker binding capacity of the secondary carrier. While investigating this problem, it was found incidentally that the electrophoretic pattern of the serum obtained from trypan blue-treated animals differed from that of control animals. This fact is of particular interest in considering protein metabolism under these conditions and indicates there might be synthesis of an abnormal protein in response to trypan blue treatment. This paper presents the results of a preliminary study of this abnormal protein component of serum.

Materials and methods. Seventy-nine male Holtzman rats, weighing 180-250 g, were used. Four ml of 0.5% saline solution of trypan blue were injected intraperitoneally once or twice daily for up to 6 days. Blood was taken at various intervals after the last injection of trypan blue. Serum was developed by the standard paper-electrophoretic method in a Spinco apparatus. Boric buffer (pH 7.5), barbital buffer (pH 8.6) and boric-borax buffer (pH 9.2) were used. The paper strip was stained with Spinco Dye B-1. Density of the strips was measured in a Spinco Analytrol to determine relative concentration of each component.

Results. An abnormal band between α-1 and α-2 globulin was consistently found in all trypan blue treated animals (Fig. 1). The abnormal band appeared during electrophoresis at pH 8.6 and 9.2, while it did not separate from α-2 globulin at pH 7.5, though its presence was apparent from dense staining in a portion of the α-2 globulin when compared to normal serum. The best separation was obtained at pH 9.2, the abnormal protein lying midway between α-1 and α-2 globulin. Trypan blue itself stayed at the origin with all 3 variations of pH. This probably indicates that trypan blue is absorbed by the paper rather than that trypan blue remained unbound to protein in the serum since the dye was precipitated almost completely from serum by 0.5% trichloroacetic acid, leaving a clear supernate. The well known contaminant red dye in trypan blue migrated only a short distance on the paper.

The abnormal protein band could be detected in the serum 1, 6, 12, and 24 hours after a single injection of trypan blue *in vivo*. Its disappearance from the blood was not studied extensively, but on one occasion it was still present 5 days after a single injection of trypan blue. This component was present in a greater amount than was α-2 globulin

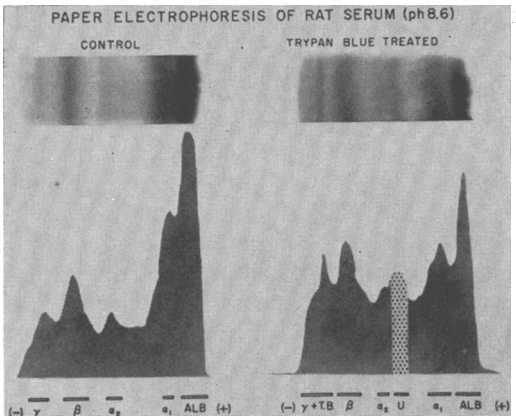

FIG. 1. In trypan blue-treated animal, 4 ml of 0.5% trypan blue in physiological saline were inj. intraper.; in the control, 4 ml physiological saline were inj. in same manner. Twenty-four hr later, blood was taken and serum developed for 16 hr. Barbital buffer pH 8.6, mA = 7.5. The unknown compound appeared between α_1 and α_2 globulin. Trypan blue remained at the origin. ALB = albumin, α_1 = α_1 globulin, α_2 = α_2 globulin, U = unknown component, β = β-globulin, γ = γ-globulin, T.B. = trypan blue.

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TABLE I. Protein Fractions in Serum of Trypan Blue-Treated and Normal Animals. Determined by paper electrophoresis in barbital buffer, pH 8.6. Development was for 16 hours at 7.5 mA or 20 hours at 10 mA.

Groups	No. of animals	% Albumin	% Globulin α-1	% Globulin α-2	% Globulin β	% Globulin γ	% Unknown compound
Normal controls	6	30.1 ± 1.4†	21.6 ± 1.2	8.7 ± .5	20.6 ± 1.4	12.4 ± .8	* 6.7 ± .2
1 hr‡	6	22.0 ± 2.9	22.8 ± 1.0	8.7 ± .4	20.2 ± 2.0	14.6 ± .6	11.7 ± .8
6 " ‡	5	22.6 ± .7	23.5 ± 1.2	8.2 ± 1.1	18.7 ± 2.5	15.8 ± 2.3	11.3 ± .2
24 " ‡	5	23.3 ± 1.9	18.7 ± 2.0	8.6 ± .9	23.7 ± 1.1	13.9 ± .5	11.8 ± .7
4 days§	6	24.8 ± .4	19.5 ± .8	8.2 ± .5	19.6 ± .7	11.6 ± .6	16.3 ± .7
6 " §	6	17.5 ± 1.1	25.0 ± .9	9.3 ± 1.1	21.7 ± 1.0	12.7 ± 1.0	13.9 ± .8

* Area which corresponded to location of abnormal protein and stained diffusely without showing any special peak was arbitrarily measured.
† Mean and stand. error.
‡ After single inj. of 4 ml of 0.5% trypan blue solution.
§ Repeated inj. of trypan blue, 4 ml every 12 hr.

even as short as 1 hour after the injection of the dye and remained fairly constant for 6 and 24 hours, but seemed to increase a little after repeated injection of the dye for 4 or 6 days (Table I). The amount of α-2 globulin, which migrated close to the abnormal component at pH 8.6, was quite constant in all of the experiments, while amount of albumin decreased markedly in all groups. It was difficult to detect any quantitative relationship between the abnormal component and proteins other than albumin, which might indicate a possible transformation of some protein(s) to the abnormal component.

Serum mixed with the same amount of trypan blue to be expected from the quantities injected *in vivo* was placed in a Dubnoff shaker for 5 hours at 39°C. No change was observed in the electrophoretic pattern, however. In another experiment serum and trypan blue were mixed and allowed to stand for 7 hours at room temperature. Again no change was produced in the electrophoretic pattern. It was also found that incubation of a serum-trypan blue mixture with liver slices did not produce this protein. Erythrocytes were hemolyzed in the presence of trypan blue and again no abnormal compound was formed.

Discussion. A correct interpretation of this phenomenon is not possible at this time. Three possible hypotheses are: (1) Trypan blue changes the electron charge of protein(s) and thus an abnormal electrophoretic pattern can be elicited. (2) Trypan blue stimulates synthesis of an abnormal protein or abnormal degradation of protein in some tissues of the body. (3) Trypan blue stimulates release of the "abnormal" protein stored in certain cells, resulting in a marked increase of its concentration in the blood.

In vitro experiments designed to discriminate among these 3 hypotheses indicated that the latter 2 appear more likely. It is not possible to say at what site this abnormal protein may be formed, nor whether this compound has any relation to thyroxine-binding globulin, but it is of interest that both are located between α-1 and α-2 globulin, and radioactive thyroxine is concentrated in this area whether the abnormal component is present or not(unpublished).

In relation to the appearance of this abnormal protein, it is of interest to recall the teratogenic effect of trypan blue(6). It might be possible that the abnormal protein metabolism produced by trypan blue has a causative role in producing abnormal fetuses, although the exact relationship between such abnormal protein metabolism and teratogenesis should be studied in pregnant animals treated with trypan blue before any conclusion can be reached.

Summary. Serum obtained from trypan blue-treated rats showed an abnormal protein band between α-1 and α-2 globulin on paper electrophoresis, which could best be separated at pH 9.2. The compound appeared within one hour and was present for several days. The quantity of this material in serum was greater than that of α-2 globulin as short as 1 hour after trypan blue injection and seemed to increase with repeated injections of trypan

blue. The compound was not formed *in vitro* by liver slices, erythrocytes or serum when incubated with trypan blue.

1. Rawson, R. A., *Am. J. Physiol.*, 1943, v138, 708.
2. Gregersen, M. I., Rawson, R. A., *ibid.*, 1943, v138, 698.
3. Robbins, J., Rall, J. E., *Rec. Prog. in Hormone Res.*, 1957, v13, 161.
4. Pitt-Rivers, R., *Ciba Foundation Colloquia on Endocrinology*, 1957, v11, 82.
5. Crispell, K. R., Coleman, J., Hyer, H., *J. Clin. Endocrinol. and Metab.*, 1957, v17, 1305.
6. Kalter, H., Warkany, J., *Physiol. Rev.*, 1959, v39, 69.

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Electrolyte Levels of Rat Salivary Secretions.* (25018)

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Recent work on rat salivary glands has revealed differences in levels of sodium and potassium in the 2 gland pairs (submaxillary-sublingual and parotid)(1). Resting submaxillary-sublingual has a high level of potassium and low sodium; resting parotid, on the other hand, exhibits a high sodium and low potassium. Stimulation of these glands over a 20 minute period, by pilocarpine, results in a loss of sodium by the parotid, while the submaxillary-sublingual loses potassium. In view of these differences in gland levels and pattern of behavior on stimulation, the electrolyte levels of submaxillary-sublingual and parotid salivas were investigated for possible correlation between electrolyte levels in the saliva and those of glands.

Methods. Adult male Long-Evans rats, weighing approximately 350 g, were fasted for 48 hours, and following light anesthetization with Nembutal, parotid or submaxillary-sublingual ducts were exteriorized, pilocarpine nitrate (2.2 mg) was subcutaneously administered, and the secretions collected from the freed ducts. To avoid contamination by residue in the ducts, the first drops were discarded. The duct was then led into a tared vessel covered with Parafilm. Following a timed collection period, usually 3 minutes, the vessel was reweighed, the secreting gland removed, and weights of both were recorded. Flow is thus expressed as mg/min or mg/min/mg of gland. An alternate collection method involved recovery of saliva from the oral cavity of animals from which one gland pair had previously been removed. Minor glands were found to contribute negligible quantities of fluid during short periods of stimulation. Electrolyte levels of each secretion were similar for the 2 collection methods during the short period of stimulation. Collection from the oral cavity was possible with restrained, unanesthetized animals; hence, this method was employed primarily to investigate possible effects of anesthetic on saliva electrolyte levels. Blood samples were removed from the jugular vein. After proper dilution of serum and salivary secretions, determinations of sodium and potassium were made by flame photometry and are expressed as milliequivalents per liter of secretion or serum.

Results. Representative values for electrolyte concentrations as well as flow rate during an initial 3 minutes of collection of submaxillary-sublingual and parotid gland secretions from the appropriate ducts are given in Table I. Sodium levels of submaxillary-sublingual secretions are low (generally less than 1/10 that of serum), and, while there may be as much as a 2-fold variation in Na levels among animals, it is apparent from Table I that such differences, in this time period, are not functions of gland size or secretion flow rate. Potassium levels of this secretion are very high, and again individual variation among animals is not correlated

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