Factors Influencing Development of Insulin-Induced Hypoglycemic Convulsions in the Rabbit. (20569)

ESTHER L. MCCANDLESS.* (Introduced by J. A. Dye.)
From the Department of Physiology, Woman's Medical College of Pennsylvania.

The introduction of the rabbit as a test animal for insulin assay was followed shortly thereafter by the recognition of the unreliability of convulsions in this species as a criterion of insulin potency(1,2). Nevertheless the rabbit has been considered satisfactory for the demonstration of insulin-induced hypoglycemic convulsions in many teaching laboratories. For the last 5 years, however, administration of "convulsive" doses of insulin in this laboratory failed to elicit seizures. Examination of possible changes in the origin and handling of rabbits in recent years yielded no definite clues to the cause of the extreme difficulty encountered. The source, breed, and diet of the animals had not been changed. That the problem was not merely a local one has been indicated by communications with workers in other parts of the country. Several reports in the literature have related variations in insulin sensitivity to breed(3), color (4), age(4), size(1) and sex(5). Acton and Bose(3) found a much higher maximal blood glucose level after epinephrine administration in resistant groups and postulated that the increased protection against the effects of insulin was due to a higher compensatory epinephrine output. Despite postulates by several workers(1,6) that differences in sensitivity were due to variations in liver glycogen, this had not been investigated in any systematic fashion. The following series of experiments was undertaken to study these aspects of the problem.

Methods. Twelve rabbits, unselected as to breed and sex and weighing approximately 2 kg initially, were used in these experiments. The animals were placed on the standard laboratory diet of rabbit pellets supplemented with fresh lettuce for 2 weeks before the experiments were started. Four procedures were carried out on each animal: 1) subcutaneous injection of regular insulin (Iletin, Lilly), 2 units/kg; 2) a repetition of this; 3) subcutaneous injection of 1:1000 epinephrine, 0.25 ml(3); 4) terminal determination of liver glycogen immediately after induction of anesthesia by nembutal injected intravenously. An interval of at least a week from the previous experiment and a 24-hour fast preceded each procedure. By keeping conditions similar in this fashion and by starting each procedure at the same time of day, it was believed that errors due to diurnal variations, etc. could be minimized. Blood samples were taken for glucose analyses before insulin or epinephrine administration and at hourly intervals thereafter. When hypoglycemic convulsions occurred, they were rapidly terminated by intravenous injection of 2 ml of 50% glucose. Glucose determinations, by the micro-method of Somogyi-Shaffer-Hartmann(7), were run on protein-free blood filtrates prepared by the method of Somogyi(8). That the glucose values obtained by this method represent true sugar was demonstrated by determining the difference in reducing substances in filtrates of rabbit blood before and after yeast fermentations. Glycogen was determined by the method of Good *et al.*(9) with corrections made for non-fermentable reducing substances.

Results and discussion. The data obtained in the above experiments divided the animals into two groups, those which exhibited convulsions at one time or another after insulin administration, the reactors, and those which did not, the non-reactors. Only 7 of the 12 rabbits treated with so-called convulsive doses of insulin responded with convulsions. Table I presents a summary of the investigation of various metabolic factors of possible significance in this problem.

Contrary to expectation and to postulates in the literature, the reactors and the non-reactors had almost identical liver glycogen values. It should be pointed out that glycogen was determined at a time of day comparable to that of the initiation of the insulin

* Present address, Department of Medicine, Jefferson Medical College.

TABLE I. Metabolic Factors of Possible Significance in Causation of Hypoglycemic Convulsions.

	Initial wt, kg*	Insulin I: Min glycemia, mg %	Insulin I: % of original	Insulin II: Min glycemia, mg %†	Insulin II: % of original†	Epinephrine: Max glycemia, mg %	Epinephrine: % of increase	Liver glycogen, terminal, g %
Reactors:								
# observations	7	7	7	6	6	7	7	7
Mean value	2.1	17.6	22.4	6.0	7.3	196.0	169.6	.31
Stand. error	.1	4.0	5.0	1.5	1.6	11.4	18.3	.14
Non-reactors:								
# observations	5	5	5	5	5	5	5	5
Mean value	2.8	27.8	37.6	29.6	35.2	195.2	174.0	.33
Stand. error	.2	8.5	13.3	5.0	4.2	6.1	33.5	.15

* Significant difference between groups, $P < .02$.
† Highly significant difference between groups, $P < .001$.

tests, and that the values represented, as nearly as possible, the stores upon which the animal could draw when hypoglycemia developed. That factors other than those described by Acton and Bose(3) were operating in the present series was indicated also by the results of epinephrine administration, which might be considered an *in vivo* measure of liver glycogen or its availability. The values summarized in the table were examined not only by statistical comparison of the means of the two groups but also in terms of possible relationships to body weight, initial blood glucose level and, in the case of epinephrine, dosage. No correlations were found. Although liver glycogen constituted a major carbohydrate reserve in the protection of the animal against hypoglycemia, it had little to do with whether convulsions occurred when hypoglycemia developed.

By definition, a low blood glucose level is a prerequisite for exhibition of hypoglycemic symptoms. It is perhaps surprising that no significant differences in the extent of hypoglycemia were apparent between the rabbits which exhibited convulsions in the first insulin test and those which did not. All animals were hypoglycemic. An increase in insulin dosage would have affected the duration rather than the depth of hypoglycemia(10). Contrary to the observation of Dotti(11) that fermentable reducing substances were absent from the blood during hypoglycemic convulsions, values of 7 and 18 mg % were found during such seizures on 2 separate occasions. However, at the time the samples were taken, convulsions *per se* had constituted an adequate stress to bring into play compensatory mechanisms; the glucose level which might have constituted the stimulus to convulsions was not assayed. One animal was outstanding in its ability to withstand glucose levels of 0 to 11 mg % for prolonged periods before convulsing. Convulsions were elicited only when the animals were hypoglycemic, but the absolute glycemic levels did not appear to determine their appearance(2).

Administration of a second dose of insulin, in most instances one week later, revealed an unexpected change in sensitivity in the reactor group. Although there were no significant differences between values obtained in the first and second tests in the non-reactor group, the reactors exhibited a marked increase in sensitivity with significant differences obtained between Insulin I and Insulin II. In each individual case not only did the blood glucose fall to a lower level but it also fell more rapidly than in the first insulin test. This was not true of the non-reactors. The increased sensitivity observed in these experiments was probably related to the phenomenon of "education" to insulin reported by Sahyun and Blatherwick(4), the observation that rabbits treated with convulsive doses of insulin at weekly intervals required lower doses of the hormone to produce seizures. The present series of experiments afforded no explanation of the phenomenon.

This increase in sensitivity produced a serious problem in the interpretation of the results of the second insulin test. Although the same factors were assayed in Insulin II as in Insulin I, highly significant differences between reactors and non-reactors were demonstrated

statistically, because the change in sensitivity had occurred in only one group. In view of the negative results of the first insulin test, however, one must conclude that the glycemic level did not become the primary determining factor in the causation of convulsions when the test was repeated.

The time at which convulsions occurred constituted another interesting aspect of the insulin tests. In 9 cases (5 rabbits) seizures took place within the 4 hours considered to be the period of maximal action of the hormone. In only 2 instances was the response delayed. One of the late reactors was the only animal which changed categories in the course of the experiments; although it did not react to the first dose of insulin, a week later a similar dose caused hypoglycemic convulsions in 6.5 hours. This might be another manifestation of increased insulin sensitivity. The other late reactor did not convulse until 7 hours after the first insulin administration, although its blood glucose level had remained below 11 mg % for 4 hours prior to this.

Several other comparisons between reacting and non-reacting animals were made besides those included in Table I. Analyses of initial blood glucose levels in the two groups revealed no significant differences; the mean value for all experiments was 76.7 mg % with a standard error of 2.0. There appeared to be no definite variation due to color or breed in these experiments; *i.e.*, 4 Chinchillas, 2 New Zealand albinos and 1 Dutch rabbit were reactors, 3 New Zealand albinos, 1 Chinchilla and 1 Himalayan were non-reactors. This observation constituted a difference from the experimental results of Acton and Bose(3) and a similarity to results reported in this country by Dotti(5). The fact that each of the 3 females studied exhibited convulsions can hardly be called significant or a confirmation of Dotti's reported sex difference in view of the small number of females involved in the present experiments. There was no observable difference in physical condition of the two groups. Coccidial nodules were present in the livers of 4 reactors and of 3 non-reactors; however none of the animals had shown other signs of the infestation.

The only other observation which had bearing on the problem was the difference in weights of the animals. Intergroup comparisons of the means of initial and terminal weights yielded P values less than 0.02 and 0.05, respectively. If these were significant differences, this observation might afford partial explanation of the difficulties encountered in our teaching laboratory. The animals used in the demonstrations were usually large rabbits which had been in the animal quarters for some time.

On the other hand, it must be admitted that none of the observations made in the course of this study fully explained why half of the present series of animals developed convulsions after insulin administration and the other half failed to show symptoms although their blood glucose levels were just as low in the first insulin test. As mentioned earlier, clearly more was involved in the elicitation of convulsions than the blood glucose level and/or its rate of fall.

The observation that the categories established in the first insulin test, reactors and non-reactors, were borne out in the second insulin test, that with only the one exception each rabbit remained in the same group, has led to the conclusion that similar factors must have been operating in both tests to determine whether a given rabbit reacted or not. Results of the experiments indicate that the factors investigated did not determine into which category each animal fell. In view of recent studies of humans responding to hypoglycemia with convulsive seizures(12), one is tempted to question whether the differences in reactivity may be a characteristic of the central nervous system and its excitability rather than a characteristic of only the metabolic-hormonal pattern of the individual. This aspect of the problem awaits investigation.

Summary. Of a group of 12 rabbits treated with "convulsive" doses of insulin, only 7 responded with convulsions. Except for the occurrence of convulsions, the reactors could not be distinguished from the non-reactors in the glycemic response to the first insulin injection, in response to epinephrine administration, or in liver glycogen. Significant differences were obtained in body weights and in the glycemic response to the second in-

sulin injection. The latter was related to an unexplained increase in insulin sensitivity in the reactors. No change in sensitivity occurred in the non-reactors.

1. McCormick, N. A., Macleod, J. J. R., Noble, E. C., O'Brien, K., *J. Physiol.*, 1923, v57, 234.
2. Clough, H. D., Allen, R. S., Root, E. W. Jr., *Am. J. Physiol.*, 1923, v66, 461.
3. Acton, H. W., Bose, J. P., *Ind. J. Med. Res.*, 1927, v15, 89.
4. Sahyun, M., Blatherwick, N. R., *Am. J. Physiol.*, 1926, v76, 677.
5. Dotti, L. B., *ibid.*, 1936, v114, 538.
6. Zeckwer, I. T., *ibid.*, 1933, v106, 273.
7. Shaffer, P. A., Somogyi, M., *J. Biol. Chem.*, 1933, v100, 695.
8. Somogyi, M., *ibid.*, 1930, v86, 655.
9. Good, C. A., Kramer, H., Somogyi, M., *ibid.*, 1932, v100, 485.
10. Reid, R. L., *Aust. J. Agric. Res.*, 1951, v2, 132.
11. Dotti, L. B., *J. Biol. Chem.*, 1934, v104, 535.
12. Greenblatt, M., Murray, J., Root, H. F., *New Eng. J. Med.*, 1946, v234, 119.

Received September 4, 1953. P.S.E.B.M., 1953, v84.

Botulinum Toxin and the Motor End Plate.* (20570)

J. H. STOVER, JR.,† M. FINGERMAN, AND R. H. FORESTER.
(Introduced by J. B. Bateman.)
From Camp Detrick, Frederick, Md.

The extreme toxicity of botulinum toxin has long been known to be due to its paralytic action on skeletal muscle. Guyton and MacDonald(1) confirmed and extended the observation that peripheral nerve conduction in toxin-paralyzed rabbits and guinea pigs was unimpaired. They also pointed out that the skeletal muscles of such organisms could contract if stimulated directly by electric shock. It seemed evident, therefore, that the toxin exerts its effect upon the neuromuscular junction by blocking conduction (a) in the terminal fibers or (b) in the motor end plate (perhaps by inhibiting the release of acetyl choline from the end plate). Burgen, Dickens, and Zatman(2) demonstrated that the amount of acetyl choline released by a nearly toxin-paralyzed muscle was greatly decreased; it was less than 15% of the original output. Brooks(3), using cat and guinea pig muscle, has presented preliminary evidence in favor of the blocking hypothesis (a). The present investigation was undertaken in the hope of adducing more evidence for the site of action of the toxin.

Materials and methods. The preparation used was the sartorius-sartorius nerve of the frog, *Rana pipiens*. The muscle was mounted in a lucite chamber and bathed with Frog Ringers. A water jacket maintained the temperature at 25°C. The muscle was stimulated indirectly via the sartorius nerve with short (0.1-0.3 msec) pulses of 1.5 V amplitude obtained from a Grass S4A stimulator. The electric reactions of the muscle were detected with a platinum-iridium, glass-coated microelectrode, led off through a single stage cathode follower amplifier mounted directly on the electrode. The signal was then carried through a differential preamplifier with voltage gain of 1000 and pass band of 0.2-40 KC and thence to a DC coupled amplifier with pass band of 0-100 KC. The signal was displayed on one gun of a 3-gun CRO. Time signals were injected on the trace of another gun, operated from a common sweep generator. The time signal generator was calibrated against a crystal controlled pulse generator. End plate potentials were located by probing when the muscle was nearly paralyzed with toxin or by poisoning the muscle with a 1 μM solution of curare

* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

† Present address: Naval Medical Research Unit No. 1, Oakland, Calif.