CCXXIII. THE DIFFERENTIAL INACTIVATION OF INSULIN.

BY BURT DAVIS, JUN., JAMES MURRAY LUCK
AND ALBERT GEORGE MILLER.

From the Biochemical Laboratory, Stanford University.

(Received July 19th, 1933.)

ALTHOUGH one of the most characteristic effects of insulin administered to the normal animal is a reduction of the blood-sugar content it has also been amply demonstrated that the concentrations of inorganic phosphate [Wigglesworth *et al.*, 1922–23] and amino-acid-nitrogen [Luck *et al.*, 1928] in the blood are also reduced. These demonstrations have been made not only with the ordinary preparations of insulin used therapeutically but also with crystalline insulin.

The object of the experiments now to be discussed was to determine whether or not these three characteristics of insulin activity are functions of the entire molecule acting as a unit or of distinct portions of the molecule, acting more or less independently. In support of the unitary hypothesis is the common belief that phosphate-lowering, following insulin, is secondary to the hypoglycaemia, or at least intimately associated with the well-recognised changes in carbohydrate metabolism. Nevertheless, no satisfactory proof of such a relationship has ever been advanced. Nor is there any evidence that the effect of insulin upon the amino-nitrogen content of the blood is dependent upon the carbohydrate changes. It occurred to us that the possible existence of an inter-relationship between these three physiological activities might be investigated directly through the use of partially inactivated preparations of the hormone.

It would be superfluous to discuss here in detail the extensive investigations of Freudenberg and associates, of Jensen, Scott, Du Vigneaud and others on insulin inactivation which have contributed much to our knowledge of the so-called "active groups" of insulin. In general, these investigations have been restricted to the chemistry of inactivation, employing as the sole criterion of activity the ability of the material to lower the blood-sugar content or, in large doses, to induce convulsions.

The present investigation is essentially an extension of such studies, the treated insulin being examined, not only in respect of its effect upon blood-sugar content but upon inorganic phosphate and amino-acid-nitrogen as well.

EXPERIMENTAL.

Inactivation of insulin. Two methods of inactivation were employed. In the first, crystalline insulin¹ was treated at 0° with 0.75*N* HCl in 75 % alcohol according to the method of Carr *et al.*, [1929]. 0.5 mg. samples were weighed

¹ We are much indebted to Dr D. A. Scott of the Insulin Laboratories, Toronto, and Prof. Du Vigneaud of George Washington University for the crystalline insulin used in this investigation.

out on a micro-balance and dissolved in 1 cc. portions of the acid-alcohol. The solutions so obtained were diluted to 12 cc. after 0, 1, 2, 3, 4, 5, 6, 7 or 8 days and thereupon injected at once into fasting rabbits.

In the second method, soft X-rays of high intensity were used as the inactivating agent. 0.2 to 0.5 cc. portions of insulin, 80 units per cc., were measured out into cylindrically ground depression slides, having a capacity of about 0.5 cc. The slides were sealed with thin mica cover slips (of known thickness) and vaseline and irradiated for periods ranging from 1 second to 2 hours.

The apparatus employed has been used by one of us (J. M. L.) in other experiments [Brown *et al.*, 1933] and was designed to give radiation of unusual intensity through a short target to specimen distance (3 cm.), silver anode, thin aluminium window (0.003 inch) and moderately high power (30 ma. at 50 kv.). The intensity as measured by an ionisation chamber approximated to 2500 Roentgen units per second¹.

Immediately after irradiation the specimens were diluted to 16–40 cc. (1 unit per cc. based on initial activity) and injected into fasting rabbits².

Experimental animals. Large female rabbits of 3–3.5 kg. weight, previously maintained upon a diet of barley and alfalfa were used. The animals were deprived of food for 2 days prior to injection.

Blood analysis. Blood samples, each of 5 cc. were collected, the first shortly before injection, the others at suitable intervals thereafter. In most of the experiments on inactivation by acid-alcohol only a single post-injection sample was drawn in order to minimise the chances of haemorrhage itself exerting an effect and thus leading to possible misinterpretation of results. In the rest of the acid-alcohol experiments, samples were drawn at 0, 1, 2, 3, 4, 5 and 6 hours. In all the X-ray experiments, the times of collection were 0, 1.5 and 3 hours.

Samples were analysed for reducing sugar, inorganic phosphate and amino-acid-nitrogen by the methods of Folin [1928], Fiske and Subbarow [1925] and Folin [1922] respectively.

RESULTS.

In Table I we have presented the results of experiments on 85 rabbits, injected with preparations of crystalline insulin which had been treated at 0° with 0.75*N* HCl in 75 % ethyl alcohol. Since it is not feasible to present the individual results, we have averaged the values within groups and reduced the averages so obtained to a common base line. This facilitates comparison.

Percentage retention of activity has also been calculated according to Freudenberg's method [Freudenberg and Dirscherl, 1928] and the values incorporated in Table I. Reference to this portion of Table I permits the conclusion that after 2 or 3 days of treatment at 0° with acid-alcohol, 25–30 % of the amino-nitrogen-lowering activity is retained, 15–50 % of the sugar-lowering activity and 60–80 % of the phosphate-lowering activity. The ability of insulin to lower the inorganic phosphate content of blood seems to be most resistant to inactivation by this means.

To obtain more frequent samples 27 additional experiments were performed, in which blood-samples were collected at 0, 1, 2, 3, 4.5 and 6 hours after

¹ We wish to acknowledge, most gratefully, the aid of Mr Morden G. Brown in operating the X-ray equipment.

² Prompt use of the irradiated insulin is important. Long-irradiated specimens, permitted to stand overnight, were found to be completely inactive with respect to amino-acid-nitrogen, sugar and phosphate. It is possible that hydrogen peroxide formed on irradiation causes this secondary destruction.

Table I. *Inactivation of insulin with acid-alcohol at 0°.*

Post-injection changes in blood composition.

Time of inactivation (days)	Number of rabbits	Three-hour post-injection values.			Percentage of initial activity retained*		
		Percentage of initial			Amino-N activity	Sugar-lowering activity	Phosphate activity
		Amino-N	Sugar	Phosphate			
0	12	80	58	76	100	100	100
1	11	89	73	74	54	64	100
2	8	95	81	80	25	52	84
3	13	93	94	83	32	14	63
5	12	—	94	92	—	16	32
7	17	100	92	92	0	22	34
8	6	98	101	85	14	0	53
9	6	93	103	98	32	0	11
Control†	11	101	99	98	—	—	—

* Activity of untreated insulin regarded as 100. The following formula was used:

$$\text{Percentage of initial activity retained} = \frac{100(a-b)}{c},$$

where a = normal concentration of amino-N, phosphate, or reducing sugar in blood; b = average concentration of given constituent 3 hours after injection with treated insulin; c = average reduction in concentration of the given constituent 3 hours after injection with untreated insulin.

† Insulin not injected.

injection. The periods of treatment with acid-alcohol were 1, 3, 5 and 8 days. The results, expressed as concentrations of the various blood constituents after 0, 1, 2, 3, 4.5 and 6 hours, were averaged by groups and plotted.

In computing the percentage retentions of activity the area over each curve was calculated and the values obtained compared with the reduction given by untreated insulin. Corrections were made, necessarily, for the slight hypoaminoacidaemia and hypophosphataemia produced by bleeding (control experiments without insulin).

The results were qualitatively the same as those presented in Table I. After 5 days of treatment with acid-alcohol only 12 % of the sugar-lowering activity was retained, in contrast with 82 % of the phosphate activity.

In Table II the results of the experiments on inactivation with soft X-rays are collected. After 15 minutes of irradiation only about 25 % of the amino-acid lowering activity remained but this fraction persisted undiminished even

Table II. *Inactivation of insulin with soft X-rays.*

Time of inactivation (mins.)	Number of rabbits	Post-injection values after 1.5 and 3 hours.						Percentage of initial activity retained		
		Percentage of initial						Amino-acid activity	Sugar activity	Phosphate activity
		Amino-acids		Sugar		Phosphate				
		1.5	3	1.5	3	1.5	3			
0	10	87	77	54	55	78	79	100	100	100
0.004-0.25	10	94	89	55	55	77	70	43	99	110
0.5-4.0	6	88	87	58	60	74	69	75	91	118
15	8	93	93	79	91	86	87	29	37	60
30	6	98	94	81	95	88	87	20	31	55
60	6	96	94	104	107	92	88	29	- 11	40
120	4	97	95	102	100	94	85	23	- 2.4	41
120*	2	96	86	106	120	91	79	45	- 24	56
120†	2	104	97	111	121	93	86	9	- 30	41

* 5 × dose.

† 10 × dose.

after 2 hours' exposure. After 30–60 minutes of irradiation the hypoglycaemic activity of the insulin was lost completely. In fact, long-irradiated specimens tended to be hyperglycaemic—a property which became more evident when the irradiated insulin was injected in massive doses. As for phosphate-lowering activity, again we observed that this property of the hormone was the most resistant towards destruction. Even after 2 hours of irradiation, from 40 to 50 % of the initial activity remained. This lowering of phosphate, obtained in the absence of hypoglycaemia, demonstrates that a substantial portion of the activity of insulin towards phosphate cannot be secondary to the carbohydrate activity of the hormone.

Table II also contains the results of several experiments in which massive doses of the long-irradiated insulin (2 hours) were injected. The object here was to determine whether these larger doses might not increase, substantially, the changes in concentration in the blood of the substances studied.

Although the amino-acid changes were inconclusive and the decrease in phosphate activity remained at 50 or 60 %, the most interesting observation was the unmistakable tendency towards hyperglycaemia. Doubtless several explanations can be advanced to account for this phenomenon. However, we are disposed to think that the irradiated insulin induces an increased secretion of adrenaline. If so, the persistent though lessened activity towards amino-acids and phosphate may be due to adrenaline.

As an incidental observation, it should be mentioned that these massive doses of irradiated insulin failed to induce convulsions.

DISCUSSION.

All the experiments described in this paper agree in showing that the phosphate-lowering activity of insulin is the most resistant to destruction by the methods of inactivation examined. They demonstrate that insulin may be differentially inactivated—in the sense that the physiological activities of insulin may be separately destroyed. A complete separation was not accomplished, inasmuch as the loss of hypoglycaemic activity was accompanied by a loss of 50 % or so of the phosphate-lowering activity. Nevertheless it is evident that these three activities of the hormone are not lost in unison as the unitary hypothesis¹ of insulin structure and function would demand. The results permit no final conclusion as to the relative ease of destruction of the amino-acid-lowering activity, although our experiments with brief periods of X-ray irradiation frequently indicated that this factor is less stable to X-rays than the carbohydrate-lowering factor.

Here it should be mentioned that controls have been run with untreated insulin administered in dosages of 1 to 1/16 unit per kg. in order to see the effect of mere insulin dilution upon the blood-constituents¹. We had in mind the possibility that the "differential" inactivation here reported might be more apparent than real; that inactivating agents, instead of affecting one kind of active group to a greater extent than another, might inactivate completely a fraction of all the molecules present—leaving the others unaltered. Such an explanation would be demanded by the unitary hypothesis. We found, however, that reduction of the quantities of insulin administered caused the activities with respect to blood-sugar and inorganic phosphate to be "diluted-out" in

¹ By the "unitary hypothesis" we mean that theory of insulin structure and function which postulates that the various physiological activities of insulin are the property of a single active "centre" or "unit."

² The experiments with these lower dosages of insulin were made by Mr Walton Van Winkle.

unison. These results will be included in a later paper of this series and need not be discussed further at this time. There was no indication, however, that low doses of insulin (comparable with a mixture of active and inactive molecules resulting from inactivation) would lower blood-phosphate without causing hypoglycaemia.

With respect to the X-ray experiments it is pertinent to mention that earlier attempts at inactivation (sugar-lowering activity) with X-rays have given only negative results. Den Hoed *et al.*, [1929] irradiated specimens for $6\frac{3}{4}$ hours from a tube fed by a current of 180 kv. and 2 ma. Since the target-to-specimen distance was 24 cm. the intensity of the incident radiation must have been only about $1/64$ as great as that employed in our experiments. We found that an irradiation time of 30 minutes was necessary to bring about an appreciable reduction of the sugar-lowering activity of insulin. Under the conditions of the experiments of den Hoed *et al.* an irradiation time of 32 hours would be required to produce a comparable effect with radiation of the same wave-length. It is apparent then that the negative results obtained in earlier experiments are due, in large part, to insufficient radiation [*cf.* Nitzescu, 1924].

Inactivation by ultra-violet radiation has been reported by several investigators [den Hoed *et al.*, 1929; Kuhn *et al.*, 1931; Freudenberg *et al.*, 1930; Ellis, 1925; Freudenberg and Eyer, 1932]. Maximum absorption and inactivation are obtained by radiation of wave-length 2750 Å. [Kuhn *et al.*, 1931; Freudenberg and Eyer, 1932]. This corresponds with the principal absorption band of tyrosine and cystine and suggests that either or both of these amino-acids are so-called "active centres." But since the absorption of X-rays is probably atomic rather than molecular, there is little likelihood of the same mechanism operating in our experiments. It would be unprofitable, therefore, without many more data, to attempt an explanation of the mode of X-ray inactivation.

SUMMARY.

1. The inactivation of insulin by acid-alcohol and by soft X-rays has been studied.
2. In both cases the phosphate-lowering activity of insulin was the most resistant to destruction.
3. Massive doses of insulin inactivated by X-rays produced hyperglycaemia; about 50 % of the phosphate-lowering activity was retained.
4. It is concluded that insulin owes its multiple activity to a number of "active groups," "centres," or "units" which vary in stability towards inactivating agents.

REFERENCES.

- Brown, Luck, Sheets and Taylor (1933). *J. Gen. Physiol.* **16**, 397.
Carr, Culhane, Fuller and Underhill (1929). *Biochem. J.* **23**, 1010.
den Hoed, de Jongh and Peek (1929). *Biochem. Z.* **205**, 144.
Ellis (1925). *Amer. J. Physiol.* **73**, 530.
Fiske and Subbarow (1925). *J. Biol. Chem.* **66**, 375.
Folin (1922). *J. Biol. Chem.* **51**, 377.
— (1928). *J. Biol. Chem.* **77**, 421.
Freudenberg and Dirscherl (1928). *Z. physiol. Chem.* **175**, 1.
— — and Eyer (1930). *Z. physiol. Chem.* **187**, 89.
— — and Eyer (1932). *Z. physiol. Chem.* **213**, 226.
Kuhn, Eyer and Freudenberg (1931). *Z. physiol. Chem.* **202**, 97.
Luck, Morrison and Wilbur (1928). *J. Biol. Chem.* **77**, 151.
Nitzescu (1924). *Klin. Woch.* **3**, 2343.
Wigglesworth, Woodrow, Smith and Winter (1922-23). *J. Physiol.* **57**, 447.