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The Influence of the Induction of Alloxan-Diabetes on the Incorporation of Amino Acids into Protein of Rat Diaphragm

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Insulin *in vitro* stimulates the incorporation of labelled amino acids into protein of isolated diaphragm from the normal rat (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958; Wool & Krahl, 1959) and from the hypophysectomized or adrenalectomized rat (Kostyo, 1959; Manchester, Randle & Young, 1959). On the basis of these and other observations, it is to be expected that the rate of incorporation of labelled amino acids into protein of isolated diaphragm from the alloxan-diabetic rat would be less than that of diaphragm from the normal rat, and that treatment of the alloxan-diabetic rat with insulin would restore the rate of incorporation to normal. This possibility, in both the newly diabetic and the 'long-term' diabetic animal, has now been investigated. The ability of plasma from rats, in various stages of alloxan-diabetes, to stimulate the uptake of glucose and incorporation of amino acid into protein by diaphragm from normal rats has also been studied.

MATERIALS AND METHODS

Animals. Albino Wistar female rats of about 130 g. wt. were used throughout. They were fed *ad libitum* a standard laboratory diet (Bruce & Parkes, 1949) and had free access to water at all times.

Alloxan-diabetes. Diaphragm was taken from rats at two widely different periods after they had been treated with alloxan. What are termed 'short-term' alloxan-diabetic rats had received alloxan 2 days (48 hr.) before removal of the diaphragm; 'long-term' alloxan-diabetic rats had received alloxan at least 2 weeks previously. In both instances the rats were starved 24 hr. before a subcutaneous injection of alloxan was given [alloxan monohydrate, British Drug Houses Ltd., 200 mg./kg. in 1% (w/v) NaCl]. Under these conditions an intense hyperglycaemia developed, and 48 hr. after the administration of alloxan the blood sugar usually exceeded 500 mg./100 ml. even though food had been withdrawn 24 hr. previously.

No deaths usually occurred within 48 hr. of the administration of alloxan, but unless the rats were given insulin daily for at least a week after the injection of alloxan, many deaths took place within 2–7 days of this treatment. The following procedure was therefore adopted for the preparation of 'long-term' diabetic animals: each animal received 2 units of protamine–zinc (PZ) insulin (Burroughs Wellcome and Co. Ltd.) subcutaneously on the evening of days 2–7 after treatment with alloxan. On days 8–10 the daily dose of insulin was decreased to 1 unit and on days 11–12 to 0·5 unit. During the period of days 2–10 inclusive, drinking water was replaced by 1% (w/v) glucose solution in order to prevent hypoglycaemia. After day 12, treatment with insulin was discontinued unless the animal showed signs of impending diabetic coma, when soluble insulin (1 unit/day) was administered for a day or two. This procedure made possible the survival, 14 days after the administration of alloxan, of about 60% of the number treated. All these animals when deprived of insulin for 2 or more days had non-fasting blood-sugar levels above 500 mg./100 ml., but after the withdrawal of food the blood sugar dropped to about 215 mg./100 ml. (range 100–500 mg./100 ml.). At

this time they possessed very little subcutaneous and retroperitoneal fat.

Hypophysectomy. Rats were hypophysectomized by the para-pharyngeal route under ether anaesthesia. Success of the operation was judged, 10 or more days later, by failure of the animals to gain weight. Hypophysectomized animals, which had been treated with alloxan (200 mg./kg. subcutaneously), as well as control hypophysectomized animals, were given 1% glucose solution to drink instead of water.

Adrenalectomy. Bilateral adrenalectomy was carried out under ether anaesthesia through a mid-line dorsal incision. The adrenalectomized rats were given 1% NaCl to drink. Alloxan (200 mg./kg.) was injected subcutaneously 48 hr. after adrenalectomy. As with the hypophysectomized rats, the alloxan-treated adrenalectomized rats and the control adrenalectomized animals were given 1% glucose solution to drink.

Incubation of diaphragm and preparation of protein samples. The methods for the removal and incubation of diaphragm, and the preparation and assessment of the content of radioactivity of samples of protein, were those described by Manchester & Young (1958). Diaphragm (2 hemidiaphragms in 2 ml.) was incubated for 2 hr. at 37° in the bicarbonate-buffered medium of Gey & Gey (1936), gassed with $O_2 + CO_2$ (95:5), usually in the presence of 1 μmole/ml. of [1-^{14}C]glycine (specific activity, about 400 μc/m-mole). A radioactive protein hydrolysate, prepared by the acid hydrolysis of protein containing a large number of ^{14}C-labelled amino acids which had been obtained from *Chlorella* grown in the presence of $^{14}CO_2$, was used at a concentration of about 0·01 mg./ml. and a specific activity of about 37 μc/mg. Radioactive materials were obtained from The Radiochemical Centre, Amersham, Bucks. In some instances glucose was added to the medium to give a final concentration of 2·5 mg./ml.

Adrenaline. Adrenaline (British Drug Houses Ltd.) was dissolved in a small quantity of 0·1 N-HCl and diluted with Gey buffer to a concentration of 1 μg./ml.

Measurement of glucose uptake by diaphragm. Uptake of glucose by diaphragm was measured as the amount of glucose which disappeared from the medium. Glucose was determined by the colorimetric method of Somogyi (1945).

Blood plasma. Blood was collected from rats, after decapitation, in heparinized beakers and centrifuged. For the estimation of insulin activity pooled blood plasma, usually from six rats, was diluted with Gey buffer to 25%, and glucose added to the diluted plasma in such amount as to make the final glucose concentration 2·5 mg./ml. The method was essentially that of Randle (1956).

It has been reported (Bornstein & Park, 1953; Whitney & Young, 1957) that blood plasma from alloxan-diabetic rats, in contrast to that of normal rats, does not stimulate the uptake of glucose by isolated diaphragm from normal rats. But if the plasma is frozen and thawed three times insulin activity may appear (Randle, 1955; Hendley, Bregman & Krahl, 1957). Plasma of alloxan-diabetic rats may therefore contain insulin which is rendered biologically inactive by the presence of an antagonist. Freezing and thawing, by making the postulated inhibitor ineffective, enables the insulin present in the plasma to be detected by its effect on the uptake of glucose by diaphragm from the normal rat. Frozen and thawed plasma was prepared by freezing the fresh plasma in a mixture of acetone and solid CO_2, and thawing at 37°, three times altogether.

Acid-ethanol extracts of precipitated plasma proteins contain material with insulin-like activity which is believed to be at least a significant proportion of the total insulin present in the plasma (Taylor, 1959). We have used the method of Taylor (1959) to determine the amount of extractable insulin in plasma from alloxan-diabetic rats. To a sample of plasma (5 ml.) an equal volume of 10% (w/v) trichloroacetic acid was added. The precipitate was collected by centrifuging and washed with 5 ml. of 10% trichloroacetic acid. It was then extracted twice with about 5 ml. of acid–ethanol [conc. (12 N) HCl–ethanol–water 3:15:5]. The combined extract was dialysed against distilled water and freeze-dried. The resulting material was dissolved in 20 ml. of Gey buffer.

Statistics. Individual values for incorporation of radioactivity (counts/min.) or glucose uptake (mg. of glucose/g. of wet tissue/hr. for different pairs of hemidiaphragms incubated under identical conditions) were grouped, averaged and the standard error of the mean was determined. The significance of the difference between means was assessed on the basis of Student's *t* test.

RESULTS

Effect of plasma from alloxan-diabetic rats on the uptake of glucose and incorporation of amino acid by normal diaphragm

Two days after the subcutaneous administration of a diabetogenic dose of alloxan the plasma from these animals, unlike that of normal rats, possessed no detectable insulin-like activity when tested by its effect either on the uptake of glucose by isolated diaphragm from the normal rat, or on the incorporation of [^{14}C]glycine into protein of isolated diaphragm from the normal rat (Table 1). Freezing and thawing of the plasma three times exerted no significant effect on the results of such tests (Table 1).

Plasma from the 'long-term' alloxan-diabetic rat also had no observable effect on the uptake of glucose by isolated diaphragm from the normal rat, in contrast with plasma from the normal rat. This was true for untreated plasma and for plasma which had been frozen and thawed three times (Table 2). Untreated plasma nevertheless stimulated incorporation of [^{14}C]glycine into protein of isolated diaphragm from the normal rat, though to a much less extent than did plasma from the normal rat; this effect was not altered by freezing and thawing three times (Table 2).

These results suggest that plasma from either 'short-term' or 'long-term' starving diabetic rats contains little or no detectable insulin nor does it contain insulin, the activity of which is masked by the sort of insulin antagonist which is inactivated by freezing and thawing. To test this inference the extraction of insulin-like material by acid–ethanol was attempted. No insulin activity was found in material extracted thus from plasma of 'long-term'

Table 1. *Effect of blood plasma from 'short-term' alloxan-diabetic rats on the uptake of glucose and incorporation of [^{14}C]glycine into protein by the isolated diaphragm from the normal rat*

All the rats used to provide plasma were starved 24 hr. before being bled. Each result is the mean value, together with the standard error of the mean, for six experiments with six pairs of hemidiaphragms. The values of P for differences which appear possibly to be significant on inspection are indicated.

Expt. no.	Addition to the medium	No. of donor rats providing plasma	Glucose uptake (mg./g. wet wt./hr.)		Radioactivity in diaphragm protein (counts/min./disk)	
1	Plasma from normal rats	3	4·02±0·22	$P < 0·001$	396± 6	$P < 0·1 > 0·05$
	Buffer (control)	0	2·55±0·16		337±27	
	Plasma from diabetic rats*	6	2·38±0·24		289±27	
	Diabetic plasma frozen and thawed three times	6	2·21±0·11		305±23	
2	Buffer (control)	0	2·46±0·15		207± 7	
	Plasma from diabetic rats†	12	2·54±0·11		201±10	
	Diabetic plasma frozen and thawed three times	12	2·78±0·13		187±11	

* Mean fasting glucose content of pooled plasma from six alloxan-diabetic rats was 1080 mg./100 ml.
† Mean fasting glucose content of pooled plasma from twelve alloxan-diabetic rats was 885 mg./100 ml.

Table 2. *Effect of blood plasma from 'long-term' alloxan-diabetic rats on the uptake of glucose and incorporation of [^{14}C]glycine into protein by the isolated diaphragm from the normal rat*

All the rats used to provide plasma were starved 24 hr. before being bled. Each result is the mean value, together with the standard error of the mean, for six experiments with six pairs of hemidiaphragms. The values of P for differences which appear possibly to be significant on inspection are indicated.

Addition to the medium	No. of donor rats providing plasma	Glucose uptake (mg./g. wet wt./hr.)		Radioactivity in diaphragm protein (counts/min./disk)	
Plasma from normal rats	3	4·20±0·11	$P < 0·001$	318±6	$P < 0·001$
Buffer (control)	0	3·22±0·10		245±9	
Plasma from diabetic rats*	6	3·23±0·10		272±6	$P < 0·05$
Diabetic plasma frozen and thawed three times	6	3·22±0·09		280±9	$> 0·02$
					$P < 0·05 > 0·02$

* Blood-sugar values for the six alloxan-diabetic rats used as blood donors: fasting 102–340 mg./100 ml. (mean 171 mg./100 ml.); non-fasting 500–700 mg./100 ml.

Table 3. *Effect of material extracted by acid–ethanol from precipitated proteins of blood plasma from normal, 'short-term' and 'long-term' diabetic rats on the uptake of glucose and incorporation of [^{14}C]glycine into protein by isolated rat diaphragm from the normal rat*

All the rats used to provide plasma were starved 24 hr. before being bled. Each result is the mean value, together with the standard error of the mean, for six experiments with six pairs of hemidiaphragms. The values of P for differences which appear possibly to be significant on inspection are indicated.

Expt. no.	Addition to medium of protein extracted from plasma of	No. of donor rats providing plasma	Glucose uptake (mg./g. wet wt./hr.)		Radioactivity in diaphragm protein (counts/min./disk)	
1	Normal rats	3	3·84±0·15	$P < 0·001$	493±26	$P < 0·02 > 0·01$
	None (control with medium alone)	0	2·59±0·30		407±12	
	Short-term diabetic rats*	6	3·68±0·19	$P < 0·02 > 0·01$	458±23	$P < 0·1 > 0·05$
	Long-term diabetic rats†	6	2·64±0·18		389±14	
2	None (control with medium alone)	0	2·46±0·15		207±7	
	Short-term diabetic rats‡	12	2·75±0·22		212±8	

* Mean fasting glucose content of pooled plasma from six short-term alloxan-diabetic rats was 365 mg./100 ml.
† Mean fasting glucose content of pooled plasma from six long-term alloxan-diabetic rats was 472 mg./100 ml.
‡ Mean fasting glucose content of pooled plasma from twelve short-term alloxan-diabetic rats was 885 mg./100 ml.

Table 4. *Comparison of the incorporation of [^{14}C]glycine into protein, the uptake of glucose and the effect of insulin (0·1 unit/ml.) on these processes, by isolated diaphragm from the normal and the 'long-term' alloxan-diabetic rat*

Number of pairs of hemidiaphragms used is shown in parentheses. The values of P for differences which appear possibly to be significant on inspection are indicated. The effect of insulin is in every case significant ($P < 0·001$).

Diaphragm from	Radioactivity in diaphragm protein (counts/min./disk)			Glucose uptake (mg./g. wet wt./hr.)		
	No insulin in medium	Insulin added	Difference	No insulin in medium	Insulin added	Difference
No glucose added to the medium						
Normal rats (6)	498±23	752±17	254	—	—	—
Alloxan-diabetic rats (6)	399±29	675±46	276	—	—	—
	$P < 0·02 > 0·01$					
Glucose added to the medium						
Normal rats (6)	490±26	798±69	308	3·04±0·16	6·01±0·32	2·97
Alloxan-diabetic rats (6)	406±21	676±41	270	2·45±0·31	5·82±0·34	3·37
	$P < 0·05 > 0·02$					
Pooled results						
Normal rats (12)	494±17	775±34	281	—	—	—
Alloxan-diabetic rats (12)	402±17	676±29	274	—	—	—
	$P < 0·001$	$P < 0·05 > 0·02$				

alloxan-diabetic rats, in contrast with results with plasma from normal rats, either with respect to uptake of glucose or incorporation of [^{14}C]glycine into protein by isolated diaphragm from normal rats (Table 3). When one sample of blood plasma from 'short-term' alloxan-diabetic rats (mean fasting plasma glucose 365 mg./100 ml.) was treated thus, the extracted material effected a significant stimulation both of the uptake of glucose and of the incorporation of [^{14}C]glycine into protein by normal rat diaphragm, but with material from another sample (mean fasting plasma glucose 885 mg./100 ml.) no detectable insulin activity was found (Table 3).

Incorporation of [^{14}C]glycine by diaphragm from alloxan-diabetic rats

Incorporation of [^{14}C]glycine into protein of the isolated rat diaphragm of 'long-term' alloxan-diabetic rats, none of which had received insulin for at least 3 days, was less than that with diaphragm from normal rats, though the effect of insulin *in vitro* was normal (Table 4). The basal uptake of glucose by diaphragm from the 'long-term' alloxan-diabetic rat was slightly, though not significantly, less than by diaphragm from the normal rat, but in the presence of insulin the total glucose uptakes were similar (Table 4).

As might be expected, treatment of 'long-term' alloxan-diabetic rats with insulin (1 unit/day for 5 days) before removal of the diaphragm raised the rate of incorporation of [^{14}C]glycine into protein of the isolated diaphragm (Table 5). Administration of a single dose of insulin (1 unit) to the intact rat 30 min. before removal of the diaphragm also stimulated the incorporation of ^{14}C from a mixture of ^{14}C-labelled amino acids into protein of the isolated diaphragm (Table 6).

As judged by the fasting blood-sugar level our rats were acutely diabetic 48 hr. after treatment with alloxan. Rather surprisingly, however, incorporation of [^{14}C]glycine into protein of diaphragm from these animals was above normal, although the uptake of glucose was significantly less than that found with diaphragm from normal rats (Table 7). The results with respect to incorporation were similar in the presence or absence in the medium of glucose (Table 7). These results with 'short-term' alloxan-diabetic rats contrast greatly with those obtained with diaphragm from 'long-term' alloxan-diabetic rats (Table 4). With diaphragm removed only 12 hr. after the administration of alloxan the uptake of glucose and the incorporation of glycine *in vitro* were a little lower, though not significantly so, than with diaphragm from the normal rat (Table 8).

Administration of alloxan to rabbits brings about a rise in blood sugar which is succeeded by

hypoglycaemia (Jacobs, 1937; Hughes, Ware & Young, 1944). Hughes *et al.* (1944) concluded that the hypoglycaemic action of alloxan could result from the liberation of pre-formed insulin by necrotic pancreatic-islet cells. A hypersecretion of insulin in the early stages after administration of alloxan might explain the increased rate of incorporation of [^{14}C]glycine by diaphragm *in vitro* 48 hr. later. But if this were so, a rise and not a depression of glucose uptake would be expected, and a depression was in fact found (Table 7). However, any growth hormone secreted as a result, direct or indirect, of the administration of alloxan might depress the uptake of glucose by diaphragm (Park, Brown, Cornblath, Daughaday & Krahl, 1952) and stimulate incorporation of amino acids (Kostyo & Knobil, 1959; Manchester *et al.* 1959). It was therefore thought to be of interest to ascertain the effect of the 'short-term' administration of alloxan to the hypophysectomized rat on glycine incorporation by diaphragm *in vitro*. These experiments were also extended to include adrenalectomized animals.

Incorporation of [^{14}C]glycine into protein of isolated diaphragm from the hypophysectomized rat 48 hr. after injection of alloxan is greater than that found with diaphragm from the untreated hypophysectomized rat (Table 9), but incorporation into diaphragm from the adrenalectomized rat is not changed 48 hr. after administration of alloxan (Table 9). These results suggest that the enhanced rate of incorporation of [^{14}C]glycine into protein of diaphragm, observed 48 hr. after the administration of alloxan, is not the result of any change of activity of the pituitary gland, but is dependent upon the presence of the adrenal glands. Since the administration of cortisol to a rat results in a depression of the rate of the incorporation of labelled amino acids into the subsequently isolated diaphragm (Manchester *et al.* 1959; Wool & Weinshelbaum, 1959), it seems unlikely that the greater incorporation, which follows 48 hr. after treatment with alloxan, results from a rise in the secretion of corticosteroids. Injection of alloxan almost certainly elicits a secretion of adrenaline by the adrenal medulla, but neither the addition of adrenaline *in vitro* (Table 10), nor pre-treatment of the rat with adrenaline (Table 10), stimulates incorporation of [^{14}C]glycine into protein of isolated normal diaphragm.

Dr A. Korner (personal communication) finds that treatment of the rat with a large dose of cortisol (1 or 2 mg./day) depresses below normal the incorporation *in vitro* of labelled amino acids into protein by a microsomal system prepared from liver. Nevertheless, treatment of the rat with a small dose of cortisol (0·1 or 0·2 mg./day) stimulates incorporation in this system. The possibility therefore arises that in some circumstances adrenal corticoids may enhance the incorporation of labelled amino acids into protein of diaphragm, and that the stimulation of incorporation which follows the administration of alloxan may result from a small rise in the rate of secretion of adrenal corticoids. We have found no experimental evidence for this, however, since treatment of intact rats with cortisol, 0·1 mg. twice daily for 48 hr. before removal of the diaphragm, has no effect on the rate of incorporation of [^{14}C]-glycine into the protein of isolated diaphragm (Table 10). But the precise conditions for success in this respect may be highly critical.

Table 5. *Effect of treatment of the 'long-term' alloxan-diabetic rat with protamine–zinc insulin (1 unit/rat/day for 5 days) on the incorporation of [^{14}C]glycine into protein of the isolated diaphragm*

The medium contained glucose (2·5 mg./ml.). Number of pairs of hemidiaphragms used is shown in parentheses. The value of P for the difference is indicated.

Rats treated with	Radioactivity in diaphragm protein (counts/min./disk)	
1% NaCl (9)	159±15	$P = 0.02$
Insulin (8)	218±11	

DISCUSSION

The insulin content of the blood plasma of alloxan-diabetic rats

The observation that blood plasma from both 'short-term' and 'long-term' alloxan-diabetic rats, unlike plasma from normal rats, does not stimulate glucose uptake by isolated diaphragm from normal rats (Tables 1 and 2) suggests that these plasmas

Table 6. *Effect of treatment of the intact rat with insulin on the uptake of glucose, and on the incorporation of ^{14}C from a mixture of ^{14}C-labelled amino acids into protein, of the isolated diaphragm*

Each figure represents the mean result for six pairs of hemidiaphragms. The values of P for the differences are indicated. Insulin (1 unit/rat) was injected subcutaneously into the rat 30 min. before removal of the diaphragm.

Rats treated with	Radioactivity in diaphragm protein (counts/min./disk)		Glucose uptake (mg./g. wet wt./hr.)	
1% NaCl	1276±67	$P < 0.001$	2·37±0·26	$P < 0.001$
Insulin	1908±66		5·60±0·49	

Table 7. *Comparison of the incorporation of* [^{14}C]*glycine into protein, the uptake of glucose, and the effect of insulin* (0·1 *unit/ml.*) *on these processes, by isolated diaphragm from the 'short-term' alloxan-diabetic rat*

Number of pairs of hemidiaphragms used is shown in parentheses. The values of P for differences which appear possibly to be significant on inspection are indicated. The effect of insulin is significant in every case ($P < 0·05$).

Diaphragm from	Radioactivity in diaphragm protein (counts/min./disk)			Glucose uptake (mg./g. wet wt./hr.)		
	No insulin in medium	Insulin added	Difference	No insulin in medium	Insulin added	Difference
No glucose added to the medium						
Normal rats (6)	241±17	395±23	154	—	—	—
Alloxan-diabetic rats (6)	336±21	450±42	114	—	—	—
	$P < 0·02 > 0·01$			$P < 0·001$		
Glucose added to the medium						
Normal rats (6)	303±9	446±18	143	2·78±0·05	6·21±0·15	3·43
Alloxan-diabetic rats (6)	467±33	592±20	125	2·15±0·09	5·57±0·24	3·42
	$P < 0·001$					
Pooled results						
Normal rats (12)	272±8	421±14	149	—	—	—
Alloxan-diabetic rats (12)	401±21	521±22	120	—	—	—
	$P < 0·001$	$P < 0·001$				

contain much less insulin than does the plasma of normal rats. Moreover, no insulin-like material, extractable by acid–ethanol, could be detected in plasma of both 'short-term' and 'long-term' alloxan-diabetic rats with a high fasting blood-glucose level, either on the basis of stimulation of the uptake of glucose or on that of the incorporation of glycine into protein of isolated diaphragm from normal rats (Table 3). Nevertheless, such plasmas probably contain some insulin, since the depancreatized rat, completely deprived of insulin, survives less than 48 hr. (Folgia, 1944). Evidence for the presence of some insulin in the blood plasma of alloxan-diabetic rats is provided by the observation of the effect of their plasma on the incorporation of [^{14}C]glycine into protein of isolated diaphragm from normal rats, but although a significant stimulation of incorporation is produced by plasma from the 'long-term' alloxan-diabetic rat (Table 2), the magnitude of this stimulation is less than that produced by plasma from normal rats.

That no additional insulin activity appears when plasma is frozen and thawed three times (Tables 1 and 2) suggests that any putative antagonists to insulin, which might mask the presence of quantities of insulin that can be detected by use of the rat

Table 8. *Uptake of glucose and incorporation of* [^{14}C]*-glycine into protein by isolated rat diaphragm, 12 hr. after administration of alloxan to the rat*

Each figure represents the mean result for six pairs of hemidiaphragms. The differences are not significant.

Rats treated with	Radioactivity in diaphragm protein (counts/min./disk)	Glucose uptake (mg./g. wet wt./hr.)
1% NaCl	248±12	2·16±0·17
Alloxan	226± 7	1·85±0·07

Table 9. *Incorporation of* [^{14}C]*glycine into protein of isolated diaphragm from hypophysectomized and adrenalectomized rats, 48 hr. after administration of alloxan*

The medium contained no added glucose. Number of pairs of hemidiaphragms used is shown in parentheses. The value of P for a difference which appeared to be possibly significant on inspection is indicated.

Animals	Radioactivity in diaphragm protein (counts/min./disk)	
Hypophysectomized rats treated with		
1% NaCl (6)	234± 9	$P < 0·05 > 0·02$
Alloxan (5)	282±17	
Adrenalectomized rats treated with		
1% NaCl (6)	311± 5	
Alloxan (6)	314±12	

Table 10. *Effect of adrenaline and of cortisol acetate on the incorporation of [^{14}C]glycine into protein of isolated rat diaphragm*

Each figure represents the mean result for six pairs of hemidiaphragms. The difference between means is not significant.

Expt. no.	Addition to the medium	Rats treated with	Radioactivity in diaphragm protein (counts/min./disk)	Glucose uptake (mg./g. wet wt./hr.)
1	No addition	—	258±8*	—
	Adrenaline†	—	251±9	—
2	No addition	1% NaCl	258±11*	1·82±0·09
	No addition	Adrenaline‡	231±13	1·91±0·12
3	No addition	1% NaCl	283±7§	—
	No addition	Cortisol acetate‖	288±10	—

* Medium contained glucose (2·5 mg./ml.). † (1 μg./ml.).
‡ Adrenaline (0·05 mg.) subcutaneously 1 hr. before removal of the diaphragm.
§ Medium contained no added glucose. ‖ 0·1 mg./rat twice daily for 2 days.

diaphragm *in vitro*, are not inactivated by freezing and thawing. Any such antagonists must differ from the inhibitor which appears in the blood of the cat when the animal is treated with growth hormone (Randle & Young, 1956) and are unlikely to be lipoproteins of the sort described by Bornstein (1953). Since Krahl, Tidball & Bregman (1959) have shown that a lipoprotein fraction, obtained from the plasma of normal rats, inhibits the stimulation by insulin of the uptake of glucose by normal rat diaphragm as effectively as does lipoprotein from the serum of the alloxan-diabetic rat, there is no reason to suppose that the lipoprotein inhibitor is peculiar to the alloxan-diabetic rat.

It seems more likely that the alloxan-diabetic rat, after the first 24 hr., is suffering from an acute lack of secreted insulin rather than from a masking of the biological activity of circulating insulin by an insulin antagonist. Ash, Pennington & Reid (1959) found that the administration of butyrate raised the blood-sugar level of the insulin-treated sheep to a much greater extent than did administration of an equal amount of glucose. Such an observation suggests that fatty or keto acids may antagonize the action of insulin. During the first few days after the administration of alloxan the diabetic rat, if not protected with insulin, loses reserve fat and suffers from a ketosis which can be fatal. It may be that antagonism of the little remaining insulin by circulating fatty and keto acids explains the large number of fatalities during the first week after administration of alloxan and the insulin resistance encountered during this phase. When the body-fat reserves have been considerably depleted the danger of ketosis is lessened.

Effect of induction of alloxan-diabetes on the behaviour of rat diaphragm in vitro

The induction of alloxan diabetes is known to decrease the uptake of glucose by rat diaphragm examined *in vitro* (Krahl & Cori, 1947; Villee & Hastings, 1949). Krahl & Cori (1947) concluded that the rate of glucose uptake *in vitro* by diaphragm from alloxan-diabetic rats is depressed only when the fasting blood-sugar level is above 300 mg./100 ml. Our results are in accordance with these observations. With 'short-term' starving alloxan-diabetic rats (in which the fasting blood-sugar level is high) the uptake of glucose by diaphragm *in vitro* is much below that for diaphragm from normal rats (Table 7), whereas the uptake of glucose by diaphragm from starving 'long-term' alloxan-diabetic rats (in which the average fasting blood-sugar level is not very high) was not so clearly sub-normal (Table 4). It may be, however, that the decreased glucose uptake of diaphragm from rats with a blood-sugar level in excess of 300 mg./100 ml. is more apparent than real. Bornstein & Park (1953) found that under the conditions normally employed for pre-treatment of diaphragm in which the tissue is soaked in cold buffer for 5–10 min. it is unlikely that glucose, already present in the diaphragm, will diffuse from the tissue into the buffer. Diaphragm from hyperglycaemic rats will, therefore, carry over more glucose into the final incubation medium than will diaphragm from normal rats and this glucose will be released into the medium during incubation with shaking at 37°, giving the impression of a smaller disappearance of glucose from the medium than is really the case.

Krahl (1953) examined the incorporation *in vitro*, in a medium to which no glucose was added, of [^{14}C]glycine into glutathione and protein of liver slices, and into protein of isolated diaphragm, with starving alloxan-diabetic rats 7 days after treatment with alloxan. In these experiments incorporation was below that found for tissues for normal rats, but was raised almost to the normal value by the addition of glucose alone to the medium. The fasting blood-sugar levels of these rats ranged from 110 to 438 mg./100 ml., but no correlation was observed for individual rats between the height of the blood

sugar and the rate of incorporation *in vitro*. In agreement with some of these results of Krahl (1953) we find that incorporation of [^{14}C]glycine into protein of diaphragm from the starving 'long-term' alloxan-diabetic rat is below normal (Table 4). We also find no correlation between the blood sugar of the diaphragm donors and the incorporation of glycine. From our experience of the chronic alloxan-diabetic rat we consider that the fasting blood sugar is not a reliable or adequate measure of the intensity of the diabetes, as judged by the degree of emaciation of individual rats and the ability of their isolated diaphragms to incorporate [^{14}C]glycine. As with diaphragm from normal rats, addition of glucose to the medium does not stimulate incorporation by diaphragm from 'long-term' alloxan-diabetic animals (Manchester, 1960). [See Manchester (1960) for a discussion of the possible reasons for these results differing from those of Krahl (1953).]

The high rate of amino acid incorporation and low glucose uptake of isolated diaphragm, observed 48 hr. after the administration of alloxan to the intact rat, is difficult to explain. The high rate of incorporation could result from an excessive liberation of insulin from necrotic pancreatic-islet cells (Hughes *et al.* 1944) but the effect of any such insulin ought also to be seen on glucose uptake, and its presence in the plasma should be detectable by an effect on normal rat diaphragm. This action of alloxan is abolished by removal of the adrenal glands, but no evidence has been found to suggest that the secretion of either glucocorticoids or adrenaline is responsible for the stimulation of glycine incorporation, though it is possible that we have not achieved the right conditions for success in this respect. There remains the possibility that administration of alloxan leads to the release of androgenic material from the adrenals and that this material produces a stimulation of amino acid incorporation into protein in diaphragm. We have not as yet been able to provide experimental support for this possibility, although it has certainly not been excluded.

SUMMARY

1. The effect of different treatments of the rat on the metabolism of its diaphragm, removed and examined *in vitro*, have been studied with respect to the uptake of glucose, the incorporation of [^{14}C]-glycine into protein, and the effect of the addition *in vitro* of insulin.

2. Two days after the administration of a diabetogenic dose of alloxan the fasting blood sugar of rats was usually about 500 mg./100 ml. Blood plasma from such animals contained no detectable insulin. The incorporation of amino acid *in vitro* by diaphragm from these alloxan-treated rats was above normal.

3. Two weeks or more after diabetes-inducing treatment with alloxan the fasting blood sugar averaged 215 mg./100 ml. Plasma from these animals had no observable effect on the glucose uptake by isolated diaphragm from the normal rat. Untreated plasma and plasma which had been frozen and thawed stimulated incorporation of [^{14}C]glycine into protein of diaphragm from the normal rat, though to a less extent than did plasma from the normal rat.

4. Two days after the administration of alloxan to the intact or to the hypophysectomized rat incorporation of [^{14}C]glycine into protein of diaphragm was significantly greater than that with diaphragm from animals not treated with alloxan.

5. Two days after the administration of alloxan to the adrenalectomized rat incorporation of [^{14}C]-glycine into protein of the animal's diaphragm *in vitro* was similar to that with diaphragm from the untreated adrenalectomized rat. Treatment of the intact rat with adrenaline or with cortisol failed to mimic the effect of the administration of alloxan in stimulating the incorporation of [^{14}C]glycine *in vitro* into protein of isolated diaphragm.

6. With diaphragm from rats which had received diabetes-inducing treatment with alloxan two or more weeks previously, the basal uptake of glucose and the basal incorporation of [^{14}C]glycine were both slightly less than those for diaphragm from the normal rat, but the effect of insulin *in vitro* on these processes was normal. Treatment of the diabetic rat with insulin for 5 days raised the basal incorporation of glycine *in vitro* to about the value for diaphragm from the intact rat.

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Studies on the Chemical Basis of the Antigenicity of Proteins

3. THE ROLE OF RIGIDITY IN THE ANTIGENICITY OF POLYPEPTIDYL GELATINS*

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An investigation of the antigenicity of various polypeptidyl gelatins (Sela & Arnon, 1960*a*) showed that the enrichment with tyrosine, tryptophan or phenylalanine converted gelatin into relatively powerful antigens of narrow specificity. The attachment of cysteine to gelatin caused a limited enhancement of antigenicity. Enrichment of gelatin with alanine, lysine or glutamic acid did not increase significantly the antigenic response.

From a comparison of the antigenic specificity of several polytyrosyl gelatins (Arnon & Sela, 1960) it was concluded that the serological specificity changes strongly as a function of the tyrosine content. Thus gelatin cross-precipitated with antibodies to a derivative with a low tyrosine content, inhibited the homologous reaction of a material with a somewhat higher tyrosine content, but did not interfere at all with the serological reaction of a derivative enriched with the largest amount of tyrosine (11%). It seemed of interest to investigate the cross-reaction of the various polypeptidyl gelatins with the antiserum to gelatin. Here, again, the increase in the tyrosine content diminished considerably the extent of precipitation.

In view of the above-mentioned results we assumed that the enhancement of the antigenicity of gelatin by the attachment of the aromatic α-amino acids and of cysteine was due to an increase in the rigidity of the molecule. According to Haurowitz (1952) a rigid structure of the determinant groups is a prerequisite for antigenicity. Even though the content of sulphydryl groups in polycysteinyl gelatin did not change significantly during a period of several months, it is possible that disulphide bonds, which would increase the rigidity of the macromolecule, are formed *in vivo*. It was therefore of interest to investigate the antigenicity of polyseryl gelatin. Serine differs from cysteine only by the oxygen which replaces sulphur, and thus no cross-linking bridges may be formed as a result of the attachment of serine to gelatin. As expected, the attachment of serine did not convert gelatin into a more powerful antigen.

In order to elucidate the role of the aromatic character of the aromatic amino acids attached, in the enhancement of the antigenicity, we have now investigated the immunological activity of poly-*cyclo*hexylalanyl gelatins. This paper presents evidence that the attachment of amino acids containing the fully saturated *cyclo*hexane rings caused a strong increase in the antigenicity, as compared with the original gelatin.

* Part 2: Arnon & Sela (1960).