EXTRACTED GLUCAGON FROM DOG PANCREAS TREATED WITH ALLOXAN, SYNTHALIN A AND COBALTOUS CHLORIDE

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In a number of studies it has been repeatedly suggested that the alpha cells of the pancreatic islet would be responsible for the secretion of glucagon. While the results of histological surveys using several cytotoxic compounds were confusing, Fodden and Read (1954) extracted glucagon from rabbits previously injected with cobaltous chloride and synthalin A to support the concept of alpha cell-secretion of glucagon. They also suggested the possible difference in the mode of action of these two compounds on islet cells.

Present paper deals with the quantitative assessment of glycogenolytic activity of partially purified glucagon fractions from dog pancreas treated with alloxan, synthalin A and cobaltous chloride, along with several findings on the nature and yield of these fractions.

MATERIALS AND METHODS

Extraction of glucagon fractions: Healthy dogs weighing from 7.0 to 19.5 kg were used for the experiment. Details of preparation of starting materials for extraction were summarized in Table I***.

Dogs were sacrificed by bleeding from carotid artery under pentobarbital anesthesia. As soon as death occurred pancreatic gland was taken out and frozen immediately by placing upon solid carbon dioxide. In no case starting materials were in frozen state more than 8 hrs. before onset of extraction. To facilitate extraction the frozen materials were crashed and then minced with meat-grinder.

Method of Sutherland and du Duve (1948) was principally followed, and for further purification isoelectric precipitation and ethanol-fractionation were used. Fraction A with minor glycogenolytic activity was removed from the extract at pH 7.5. After glucagon fraction was precipitated by the addition of 1.7 volumes of absolute ethanol and 2.8 volumes of ethyl ether,

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Table 1. Treatment of dogs

Treatment	Abbreviation	No. of animals	Body weight kg	Time of sacrifice hrs.*	Weight of pancreas g
None	I	3	14.0	—	92
			13.0	—	
			13.0	—	
Alloxan 75 mg/kg, i.v. after 5 days alloxan 50 mg/kg, i.v.	A	3	11.0	48	93
			7.0	48	
			12.0	48	
Synthalin A 6 mg/kg, i.m.	S	4	8.5	24	121
			8.5	24	
			9.5	24	
			15.5	(16)	
Alloxan 75 mg/kg, i.v. after 6 days synthalin A 6 mg/kg, i.m.	S-A	3	15.5	(15)	87
			12.0	48	
			10.5	45	
Cobaltous chloride 50 mg/kg, i.v.	Co	4	19.5	(10)	123
			15.0	(12)	
			8.5	10	
			10.0	12	
Alloxan 50 mg/kg, i.v. after 3 days alloxan 100 mg/kg, i.v. after 4 days cobaltous chloride 50 mg/kg, i.v.	Co-A	3	13.9	12	86
			12.5	(6)	
			10.0	12	

* Time elapsed after the last treatment. Figures in parentheses show the time elapsed before occurrence of death due to the toxicity of agents used.

the separated precipitate was dissolved into small amount of water at pH 3.7, centrifuged, and then brought to pH 6.1–6.2 using dilute NaOH. After standing overnight in the cold, the precipitate was separated by centrifugation and dried in vacuo (Fraction C). Fraction C was then subjected to ethanol fractionation and Fraction E was obtained at ethanol concentration of 85%, after removing minute amount of Fraction D precipitated at 71% ethanol concentration. Fraction E was dried in vacuo and stored in a desiccator.

Part of Fraction E was treated with cysteine to inactivate the contained insulin by the method of du Vigneaud *et al.* (1931) and the resultant fraction was designated as Fraction F.

Not only every precipitate in dried form was weighed but protein content of less active supernatant was also checked colorimetrically by the method of Lowry *et al.* (1951) in the interest of quantitative consideration. Throughout the experiment greatest attention was paid for the reproducibility of extraction conditions, especially of temperature, duration and other practical details of each step.

Histological examination: Prior to freezing, a small piece of pancreatic tissue was cut off and was fixed in Bouin's fluid for histological examination. Gomori's chromium-hematoxylin phloxine method (Gomori, 1939) was employed for the differential staining of the islet cells.

Hyperglycemic activity test: The glycemic property of each fraction was examined by injecting intravenously into healthy normoglycemic rabbit with quantities of 2 mg (Fraction E) and 4 mg (Fraction F) per animal. The rabbit was fasted for more than 18 hrs. prior to the experi-

ment. Samples were dissolved in distilled water at pH 3 and injections were made in volume of 2 cc per animal. Blood sugar determinations were carried out by the method of Hagedorn and Jensen (1923). For comparison of various E and F fractions one and the same rabbit was used throughout the experiment. At least 4 days were elapsed between two successive tests.

Glycogenolytic activity test: "Liver slice-homogenate" method previously reported (Ui *et al.*, 1956) was used to assess glycogenolytic activities of E fractions. It was intended to minimize variation by assigning low and high doses of all fractions in question to the liver slice from one and the same rabbit on each experimental day. Low (100 μ g per incubating flask) and high doses (200 μ g per incubating flask) of six kinds of the fractions (I, A, S, S-A, Co, Co-A) along with a control for basal glucose output comprised one day's test, and each value was obtained from 5 repetitious determinations. The experiment consisted of three days repetition, thus giving 39 values with 195 determinations. All assignments were made at random.

RESULTS

Table 2 shows the dry weight of principal fractions obtained. High yields of active fractions (Fractions C and E) from the alloxan-treated (A) and the cobaltous chloride-treated (Co) dog pancreas will readily be apparent. It should be also noticed that the repeated trials to obtain F-fraction were unsuccessful in the alloxan-treated group (A). This fact suggests the presence of relatively large amount of unknown substance(s) which might be different from the glycogenolytic

Fig. 1. Electrophoretic pattern of Fraction E

Michaelis buffer, pH 2.9,
 $\mu = 0.18$ 7 mA, 60 mins.
bath temperature 8–10°C,
slit angle 60°

principle in Fractions C and E of this group, whereas rich yield of Fraction F in the cobaltous chloride-treated group (Co) suggests actual increase of the active principle. Electrophoretic patterns of these fractions support this view (Fig. 1). In the synthalin A-treated group (S), while the yield of the inactive fraction A was obviously high, that of the active fractions C and E were found to be rather low. Low yields of the active fractions C and E in the cobaltous chloride and alloxan-treated group (Co-A) were accompanied with equally low yield of inactive fraction A, without any noticeable peculiarity in the first step of extraction process.

Figures 2 and 3 show glycemic behavior of E and F fractions extracted from variously treated dog pancreas. Although these data using single rabbit for each experiment are far from quantitative, lack of hypoglycemic response in all fractions from alloxan-treated group and in all F fractions clearly demonstrates the adequacy of these treatments. Tendency of larger responses and plateau-like hyperglycemic curves in alloxan-treated groups were also noticed in accordance with the reports of previous workers (Sutherland and de Duve, 1948).

Experimental results are listed in Table 4 in form of two randomized blocks. Apparent high glucose output on the second day of experiment was certified by statistical analysis of variance ($\alpha < 0.01$).

Relation between the sums of percentage increases in glucose output and the

Table 2. Yield of principal fractions

Treatment	Starting material g	Fraction obtained (mg)			
		A	C	E	F/E used
I	92 (100)	449 (488)*	92 (100)	80 (87)	21/58 (32)
A	93 (100)	434 (467)	189 (202)	161 (173)	0/29; 0/34 (0)
S	121 (100)	716 (591)	95 (79)	66 (55)	23/40 (32)
S-A	87 (100)	395 (454)	82 (94)	71 (84)	13/40 (15)
Co	123 (100)	516 (420)	161 (131)	142 (115)	36/81 (51)
Co-A	86 (100)	234 (272)	56 (65)	42 (49)	5/20 (13)

* Figures in parentheses show the relative yield of each fraction for 100 g of starting material.

Table 3. Glycogenolytic test on the 2nd day of experiment
Glucose output (mg glucose/g liver)

Fraction E	I	A	S	S-A	Co	Co-A	Control
Low dose 100 μ g per flask	12.5	11.7	11.7	12.0	13.6	15.7	9.6
	12.3	12.3	11.2	12.0	14.1	13.3	10.1
	13.1	12.0	11.5	11.7	13.3	14.5	10.4
	13.1	12.6	12.0	12.3	13.9	14.7	9.9
	12.8	12.8	12.0	11.7	13.6	14.4	10.1
Mean	12.76	12.26	11.68	11.94	13.70	14.53	10.02
%-in-crease	27.3	22.4	16.6	19.1	36.7	45.0	
High dose 200 μ g per flask	14.9	13.1	13.9	14.9	16.5	14.7	
	15.5	12.8	14.1	14.9	16.3	14.4	
	15.2	13.3	14.1	14.7	16.5	14.4	
	15.7	13.3	14.4	14.7	16.3	14.7	
	16.0	13.3	14.7	14.1	16.5	13.9	
Mean	15.46	13.16	14.24	14.66	16.42	14.42	
%-in-crease	54.3	31.3	42.1	46.3	63.9	43.9

Fig. 2. Blood sugar response of Fraction E
Rabbit No. 8 (♂), body weight 2.2-2.5kg, 2.0 mg of each fraction was injected intravenously

Fig. 3. Blood sugar response of Fraction F
Rabbit No. 66 (♂), body weight 2.2-2.7kg, 4.0 mg of each fraction was injected intravenously

Table 4. Result of glycogenolytic assay
Percentage increase of glucose output ($\times 10$)

Dose	Days of exper.	I	A	S	S-A	Co	Co-A	Sum
Low	1	172	83	159	125	194	34	767
	2	273	224	166	191	367	450	1671
	3	156	132	26	131	146	138	729
	Sum	601	439	351	447	707	622	3167
High	1	388	325	344	328	384	167	1936
	2	543	313	421	463	639	439	2818
	3	228	126	125	213	340	77	1109
	Sum	1159	764	890	1004	1363	683	5863

Table 5. Summary of analysis of variance for 4-point parallel line assay and relative potency of each E fraction against that of intact group

Nature of variation	Degree of freedom	A	S	S-A	Co	Co-A	I
Preparation	1	*	*	—	—	—	Standard
Regression	1	**	**	**	**	—	
Parallelism	1	—	—	—	—	—	
Between dosage	3	*	**	**	**	—	
Between days	2	*	**	*	**	**	
Error	6						
Total	11						
Relative potency		0.6458	0.7204	0.8253	1.1936	—	1.0000
Fiducial limits	Lower	0.5155	0.6230	0.7910	1.0732	—	
	Upper	0.8094	0.8332	0.8609	1.3275	—	

* represents significance at 5 per cent level

** represents significance at 1 per cent level

Fig. 4. Dosage-response line of glycogenolytic assay

dosage levels (Fig. 4) suggests the applicability of parallel line assay analysis to these data with possible exception of cobaltous chloride and alloxan-treated group (Co-A).

Table 5 show the estimated relative potencies with their fiducial limits and summarized results of the analysis of variance of each sample against E-fraction from intact dogs calculated by 4-point assay method.

As was feared beforehand, the mean squares for regression in the Co-A group assay was not significantly greater than that for error, and as far as this sample was concerned parallel line assay lost its fundamental validity. In alloxan-treated (A) and synthalin A-treated (S) groups

differences of preparations was found to be significant. Strictly speaking, these also cast doubts on the validity of the assay. Nevertheless, in these cases in the interest of approximate comparison of glycogenolytic activities, relative potencies and their fiducial limits were tentatively calculated. The low figure of relative potency in alloxan-treated group (A) again suggests the presence of unknown, biologically inert substance(s) in the E-fraction of this group.

If it is justified to consider that the product of relative yield and relative potency represents the total potency of the fraction, the last column of Table 6 would give rough measure of glycogenolytic activities extracted from dog pancreas which received the several treatments cited.

Table 6. Relative index of total activity extracted from variously treated pancreas

Treatment	Estimated relative potency of Fraction E	Fiducial limits of estimated potency	Relative yield of Fraction E	Index of total activity extracted
Intact control	100	—	100	100
Alloxan	64.6	80.9 51.6	199	100—160
Synthalin A	72.0	83.3 62.3	63	40—50
Synthalin A and alloxan	82.5	86.1 79.1	97	80
Cobaltous chloride	119.4	132.8 107.3	132	140—180
Cobaltous chloride and alloxan	—	—	54	(<50 ?)

In the alloxan-treated group (A), extracted glycogenolytic activity was found to be nearly equal to or somewhat greater (up to 1.6 times) than the intact group, and this was in accordance with the findings of earlier workers. In the synthalin A-treated group (S) both relative yield and relative potency were poorer than the standard, and that in total its activity was only half as much as that of the intact group. Pretreatment with alloxan seemed to offset to some extent the glycogenolytic activity reducing power of synthalin A. In the cobaltous chloride-treated group (Co) both the relative yield and relative potency were richer than the control, and the activity totalled was 1.4 to 1.8 times over that of the intact group. Although no valid estimate of relative potency in the cobaltous chloride and alloxan-treated group (Co-A) was obtained, experimental data suggested considerably low activity, and if one takes into consideration of the low relative yield it would be fairly asserted that the total activity extracted in this group was far less than half of the intact group.

Because of obvious difficulty, quantitative evaluation of pancreatic islet cell destruction was not attempted, but from the qualitative standpoint it was seen that synthalin A was the more preferential destructive agent against alpha cells than cobaltous chloride and that the latter sometimes simultaneously affected beta cells as well as alpha cells to result in rather general and non-selective degeneration of islets. Observation of the pancreatic islet in the synthalin A-treated (S) and

cobaltous chloride-treated (Co) groups revealed various stage of destruction of alpha cells, while in both cases there were occasionally recognized almost intact islets which survived the treatment.

DISCUSSION

The data presented in this paper and in that of Fodden and Read (1954) show that extractable glycogenolytic activity from pancreas of animals treated with synthalin A is less than that of normal and that cobaltous chloride pretreatment contrarily increases hyperglycemic activity of pancreatic extracts. On the other hand, results on the effect of simultaneous treatment with alloxan were rather conflicting. Particularly low yields of active and non-active fractions and the fact that the dose-response regression was not significant might suggest a special feature of cobaltous chloride and alloxan-treated group (Co-A) in the present experiment, and further observation will be needed for the proper understanding of the contradictory findings.

Inhibition of glucagon-induced glycogenolysis by the co-existence of insulin has been discussed, and Fodden and Read relied upon this in explaining their results on the difference between cysteine-treated and non-treated extracts. Preliminary experiments in this laboratory confirmed glycogenolysis-inhibiting action of insulin, but the degree of inhibition was not so high as to fully understand Fodden's results. Their result on glycogenolytic activity of the extract from cobaltous chloride-treated rabbits, which was nearly equal to the control in contrast to the highest activity after cysteine treatment, can only be explained by the increased deposition of insulin in pancreatic islets in this experimental group. However, this is not likely because their data showed no sign of greater hypoglycemic response of this fraction, and the electrophoretic examination of the corresponding fraction in this laboratory revealed that especially rich content of insulin along with glucagon was unlikely.

As we were aware of inhibition, though not so marked, of insulin on glycogenolytic activity of glucagon, insulin-inactivated Fraction F should have used in the present experiment for comparison of glycogenolytic activity extracted from various sources. Nevertheless, we also noticed of considerable reduction of glycogenolytic activity after cysteine treatment, and it was assumed that the drastic experimental conditions of the process, *e.g.* precipitation of inactivated insulin fraction from the reaction mixture at 4 per cent concentration of hydrochloric acid, might be destructive to glucagon itself to some extent. For fear of arbitrary loss of glucagon activity by the cysteine treatment, and because Fraction F in the alloxan-treated group (A) was not obtainable, Fraction E was used for the experiment in spite of the contamination of insulin.

Attempts to correlate the amount of extracted activity from any specific endocrine gland to the secretional stasis of any specific endocrine cell of that organ will be met with various objections even if sufficient precautions were taken to the following points.

(1) Quantitative and reproducible extraction of active principle and reasonable purity of fraction to be assayed.....The two requisites are in some way con-

tradictory. Acquisition of purity of glucagon fraction in the present experiment by repeated fractionation results in accompanied loss of recovered activity, whereas assay of impure fraction is subject to inhibition by insulin and other co-existing principles. Although electrophoretic analysis revealed that the fraction employed for comparative assay were still in impure state, conciliation between purity and quantitative recovery had to be intended.

(2) Quantitative bioassay with objective validity.....Original liver slice assay of Sutherland *et al.* (1948) was subject to large 'slice to slice' variation and completion of simultaneous assays of several test samples against single standard was practically impossible. Liver slice-homogenate method reduced the number of determinations necessary to acquire reasonable precision, and the adoption of present experimental design with statistical analysis was realized (Table 3).

Not only quantitative evaluations of cell destruction caused by the cytotoxic compounds are very difficult to accomplish, but the large variation in individual susceptibility to a single injection of the so-called "specific destructive agent", which is now a well known phenomenon in the case of incidence of alloxan diabetes, obscures the relationship between specific cells of pancreatic islet and glucagon secretion. Neither the complete absence of glycogenolytic activity in the extracts of synthalin A-treated pancreas, reported by Fodden and Read, nor complete destruction of pancreatic alpha cells in this group of animals, which might correspond with each other if the concept of alpha cell secretion of glucagon was correct, were observed in the present experiment.

In considering the effect of cobaltous chloride on the pancreatic islet cells, attention should be turned to the systemic changes, caused by its marked toxicity, which might affect the pancreatic endocrine functions.

Although present experimental data also strongly suggest that alpha cell may be responsible for glucagon secretion, it would be unfair to draw any definite conclusion on this point at the present stage of investigation.

SUMMARY

Glycogenolytic activities of partially purified fraction from dog pancreas following the administration of cobaltous chloride, synthalin A and alloxan were compared using the corresponding fraction from intact dogs as a standard.

Synthalin A treatment reduced and cobaltous chloride treatment increased the extractable glycogenolytic activity in dog pancreas.

The results are discussed in comparison with that of Fodden and Read (1954) in which rabbits were used as experimental animals.

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