

STUDIES ON CRYSTALLINE INSULIN.*

IX. THE ADSORPTION OF INSULIN ON CHARCOAL.

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It was thought desirable to repeat the work of Dingemans who claims to have been successful in obtaining an insulin preparation with an activity of about 150 international units per mg. (1). Du Vigneaud, Geiling, and Eddy (2) who studied the adsorption of crystalline insulin on charcoal found that the product obtained after adsorption was not more active than the material they started with and that it also could be again obtained in crystalline form. These investigators used norit (Eastman Kodak Company) as an adsorbent. Dingemans, however, states in a report which she kindly sent us that only with a certain Dutch preparation—medicinal supranorit obtained from the Noritmaatschappij Ltd.—could positive results be obtained by her and that Merck's charcoal, for instance, was useless. This information, however, was not available to du Vigneaud, Geiling, and Eddy.

I. Adsorption of a Pyridine Precipitate on Charcoal (Supranorit).

In the beginning of the past year the adsorption of insulin on charcoal with the preparation specified by Dingemans had already been repeated in this laboratory. The procedure employed by us may be shortly outlined: 200 mg. of the pyridine precipitate obtained from the concentrated commercial solution of insulin (Squibb) by adding 13.5 per cent pyridine and evaluated at about 20 units per mg. were dissolved in 150 cc. of 0.01 N HCl, to the clear solution were added 400 mg. of charcoal (supranorit Dutch preparation), and the whole was shaken for 1 hour. After filtration the charcoal was washed with absolute alcohol and ether.

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It was found that by neutralizing the filtrate a comparatively large precipitate was formed, thus indicating that not all the insulin had been adsorbed; the precipitate was not evaluated, but contained apparently all the impurities. The dried charcoal powder was ground up with 3 cc. of 90 per cent phenol in a mortar, let stand for about 10 minutes, and then filtered with mild suction through a hardened filter. The charcoal was again mixed well twice with 3 cc. of 90 per cent phenol. The combined phenolic filtrates which contain the insulin were poured into 30 times their volume of water and stirred well until all the phenol had been dissolved in water. The insulin which flocks out was centrifuged off, and washed with absolute alcohol and ether. This preparation (Preparation A) was compared with crystalline insulin which had been evaluated at about 24 units per mg., according to the same method given in a previous paper from this laboratory (3). As can be seen from Table I, Experiments 1 and 2, this preparation was not more active than crystalline insulin but had about the same strength as crystalline insulin. The increased activity of the adsorbed product over that of the preparation started from shows that purification of insulin can be effected in this way, as has also been shown by Moloney and Findlay (4). The figures given in Table I are the average values obtained with the number of animals indicated. The animals were used in groups of four on different days according to the procedure outlined in a previous communication (3).

We thought it advisable to find out whether perhaps by a second adsorption of this product (Preparation A) on charcoal a stronger preparation than crystalline insulin could be obtained. In the final step of the purification of the Dutch insulin preparation (organon) $m/15$ disodium hydrogen phosphate solution was used as a solvent by Dingemans. She also stated that the products obtained in the final step of purification are rather unstable and have to be injected the same day when prepared in order to obtain the high values of the activity. We, therefore, also employed $m/15$ disodium hydrogen phosphate solution as a solvent and all preparations were standardized against crystalline insulin the same day immediately after having been secured. The procedure of adsorption was similar to that outlined above. We therefore give only the amounts used: 20 mg. of Preparation A, dissolved

TABLE I.

Experiment No.	Preparation.	No. of rabbits.	Average weight.	Dose per kilo.	Average of blood sugar, mg. per 100 cc.				Convulsions.
					Normal.	1½ hrs.	3 hrs.	5 hrs.	
			kg.	mg.					
1	Crystalline insulin dissolved in M/15 Na ₂ -HPO ₄ .	12	2.05	1:40	109	59	86	104	2
2	Squibb's pyridine precipitate. First adsorption on charcoal. Dissolved in M/15 Na ₂ -HPO ₄ .	12	2.07	1:40	104	61	81	101	4
3	Crystalline insulin dissolved in M/15 Na ₂ -HPO ₄ .	12	2.19	1:40	114	57	80	95	3
4	Squibb's pyridine precipitate. Second adsorption on charcoal. Dissolved in M/15 Na ₂ -HPO ₄ .	12	2.12	1:40	122	67	92	100	None.
5	Crystalline insulin kept in solution overnight. Dissolved in M/15 Na ₂ -HPO ₄ .	12	1.91	1:125	109	74			
6	Dutch insulin (preparation of Dr. Dingemans) kept in solution overnight. Dissolved in M/15 Na ₂ -HPO ₄ .	12	1.80	1:125	115	97			
7	Crystalline insulin kept in solution overnight. Dissolved in M/15 Na ₂ -HPO ₄ .	4	1.84	1:65	113	54			
		4	1.97	1:125	117	73			
8	Crystalline insulin adsorbed on charcoal kept in solution overnight. Dissolved in M/15 Na ₂ HPO ₄ .	3	1.80	1:65	114	60			
		4	2.11	1:125	101	69			

TABLE I—*Concluded.*

Experiment No.	Preparation.	No. of rabbits.	Average weight.	Dose per kilo.	Average of blood sugar, mg. per 100 cc.				Convulsions.
					Normal.	1½ hrs.	3 hrs.	5 hrs.	
9	Crystalline insulin dissolved in M/15 Na ₂ -HPO ₄ .	12	2.02	1:80	112	52	83		3
10	Pig insulin adsorbed on charcoal. Dissolved in M/15 Na ₂ HPO ₄ .	12	2.04	1:80	110	48	64		
11	Crystalline insulin dissolved in M/15 Na ₂ -HPO ₄ .	4	1.92	1:80	99	35	55		1
12	Pig insulin adsorbed on charcoal kept in solution overnight. Dissolved in M/15 Na ₂ -HPO ₄ .	4	1.94	1:80	108	59	79		

in 15 cc. of M/15 disodium hydrogen phosphate, were shaken with 20 mg. of charcoal (supranorit, Dutch brand). Charcoal powder was extracted three times with 1 cc. of 90 per cent phenol.

As can be seen from Experiments 3 and 4 (Table I) no increase in activity could be observed; the preparation obtained after the second adsorption had the same activity as the material started from and was found to be about as active as crystalline insulin.

Our findings led us to believe that we had accumulated enough evidence to show that it was not possible under the conditions described above to obtain a more active preparation than crystalline insulin, at least not from the Squibb's product. Later, however, one of us was informed by Doctor Dingemans that not all grades of the supranorit (Dutch preparation) were equally suitable for this purpose. Through the kindness of Doctor Dingemans we came into possession of some charcoal and also some insulin (organon) which according to Doctor Dingemans had been used by her with success.

II. Adsorption of Crystalline Insulin on Charcoal.

First of all we thought it of importance to see if by adsorption of crystalline insulin on this particular grade of charcoal a more active product could be obtained. On account of the stated instability of the more highly active products, the adsorbed insulin was standardized against crystalline insulin on the very day of its preparation. $m/15$ disodium hydrogen phosphate was again used as a solvent. Procedure and amounts employed were as given under the description of the adsorption of Preparation A on charcoal.

As can be seen from Tables II and III no increase of activity of the adsorbed crystalline insulin over that of the crystalline insulin started from could be found. It seems, therefore, that it is not possible to obtain a more active preparation from crystalline insulin even with the special grade of charcoal used by Dingemans.

Some crystalline insulin was sent in the fall of 1929 to Doctor Dingemans so that she might try to get a more active product from crystalline insulin. Unfortunately, we have not heard from her up to now about the outcome of her experiments. There remains, of course, still to be considered the possibility that by the process of manufacture of the Squibb's insulin the more active form of insulin, if such exists, has been destroyed or changed to a more stable but less active form. We were therefore anxious to see if we could obtain the more active preparation of Dingemans by repeating her work as closely as possible, using the *same* charcoal and the *same* insulin preparation that she did.

III. Repetition of Work of Dingemans.

2.5 gm. of insulin, organon, preparation of Dingemans which had an activity of about 2.5 units per mg. were shaken with 50 cc. of 0.01 N sodium bicarbonate solution. All the material went into solution; the pH of this solution was found to be about 4.5, which explains why all was dissolved. The pH of the solution was adjusted to about 7 by adding 0.01 N sodium bicarbonate. The precipitate, which according to Dingemans should contain nearly all the insulin whereas most of the impurities should remain dissolved, was centrifuged off, and washed with absolute alcohol and ether. About 1 gm. was obtained.

1 gm. of this purified insulin preparation was dissolved in 200 cc. of 0.01 N hydrochloric acid. The solution which was still

TABLE II.

Crystalline Beef Insulin, 1 Mg. Dissolved in 65 Cc. of M/15 Na₂HPO₄.

The actual dose (1 cc. of the preparation) contained $\frac{1}{8}$ mg. of insulin.

Date.	Rabbit No.	Weight.	Blood sugar.			
			Normal.	1 hr.	1½ hrs.	
		kg.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	
1929						
Oct. 9	1	2.38	122	71	82	Convulsions.
	2	1.40	133	56	49	
	3	2.44	127	67	71	
	4	1.55	105	51	44	
" 16	5	2.15	117	58	62	
	6	2.40	108	80	76	
	7	1.84	115	51	51	
	8	2.66	98	58	73	
" 11	9	1.98	94	53	33	Convulsions.
	10	1.55	87	51	46	
	11	1.85	98	62	60	
	12	2.10	92	58	53	
" 18	13	2.34	107	68	68	
	14	1.57	99	59	54	
	15	1.60	118	50	57	
	16	2.60	111	46	54	
" 14	17	2.10	115	42	57	
	18	1.60	112	55	51	
	19	2.04	112	71	78	
	20	1.58	112	50	39	
" 21	21	1.80	108	49	42	Convulsions.
	22	1.73	106	60	71	
	23	Died.				
	24	1.60	144	62	67	

acid after all the insulin had gone into solution was shaken with 2 gm. of medicinal supranorit (preparation of Dingemans) for 1 hour. It was found that the solution gradually became neutral,

probably due to the presence of basic metallic oxides like calcium oxide in the charcoal. We therefore used more 0.01 N hydrochloric

TABLE III.
Crystalline Beef Insulin, Adsorbed on Charcoal, 1 Mg. Dissolved in 65 Cc. of
M/15 Na₂HPO₄.

The actual dose (1 cc. of the preparation) contained $\frac{1}{5}$ mg. of insulin.

Date.	Rabbit No.	Weight.	Blood sugar.		
			Normal.	1 hr.	1½ hrs.
1929		kg.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
Oct. 16	1	2.45	103	58	67
	2	1.78	110	58	58
	3	2.50	98	66	71
	4	1.60	100	53	40
" 9	5	2.20	117	83	78
	6	2.30	124	103	101
	7	1.90	110	85	83
	8	1.91	105	76	47
" 18	9	1.97	91	25	28
	10	1.45	109	41	55
	11	1.80	97	55	57
	12	2.07	90	57	55
" 11	13	2.35	110	74	96
	14	1.73	94	71	64
	15	1.63	113	76	67
	16	2.65	96	58	65
" 21	17	2.04	133	76	73
	18	1.54	119	62	65
	19	2.03	119	78	73
	20	2.20	106	65	74
" 14	21	1.73	105	41	46
	22	1.83	108	69	68
	23	2.32	98	62	73
	24	1.51	107	33	
					Convulsions.

acid when this experiment was repeated so that the solution was always acid. The charcoal on which the insulin was adsorbed was

filtered with mild suction, and washed with absolute alcohol and ether. It was then ground up in a mortar with 5 cc. of 90 per cent phenol, and after standing for 10 minutes was filtered through a hardened filter with mild suction, and the charcoal powder was again mixed well twice with 2 cc. of 90 per cent phenol and filtered. The combined phenolic filtrates were poured into 30 times their volume of water. With the help of stirring the phenol was

TABLE IV.

Crystalline Insulin, 1 Mg. in 125 Cc. of M/15 Na₂HPO₄.

The actual dose (1 cc. of the preparation) contained $\frac{1}{125}$ mg. of insulin.

Date.	Rabbit No.	Weight.	Blood sugar.	
			Normal.	1½ hrs.
		<i>kg.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>
1929				
Nov. 7	34	1.98	95	59
	11	1.83	108	64
	15	1.67	117	69
	64	2.10	121	62
" 13	56	1.70	108	87
	11	1.92	124	74
	32	1.80	112	72
	69	1.70	107	69
" 9	60	2.00	116	75
	7	1.92	123	84
	54	1.65	130	69
" 11	17	2.05	122	74
	19	2.13	119	91
	62	1.80	120	96
	45	1.60	105	73

gradually brought into solution; the insulin was centrifuged off, and washed with absolute alcohol and ether. The insulin preparation thus obtained was adsorbed a second time on the supranorit of Dingemans in 0.01 N hydrochloric acid solution, employing the corresponding amounts of reagents. About 100 mg. of insulin were obtained after this second adsorption from the 2.5 gm. of insulin (organon) started from. The activity of this preparation which according to Dingemans should be at least as high as

that of crystalline insulin was not determined. This product was used as the starting material for the final step in the procedure employed by Dingemans for the preparation of her highly active insulin. In this final step $M/15$ disodium hydrogen phosphate was used as a solvent. We found in agreement with Dingemans that only a comparatively small amount of this product was soluble

TABLE V.

Dutch Insulin (Preparation of Dingemans) Adsorbed on Charcoal, 1 Mg. in 125 Cc. of $M/15 Na_2HPO_4$.

The actual dose (1 cc. of the preparation) contained $\frac{1}{125}$ mg. of insulin.

Date.	Rabbit No.	Weight.	Blood sugar.		
			Normal.	1½ hours.	
		<i>kg.</i>	<i>mg. per 100 cc.</i>	<i>mg. per 100 cc.</i>	
1929					
Nov. 13	34	1.95	138	87	
	12	2.13	107	72	
	15	1.65	142	101	
	64	2.10	119	99	
" 7	56	1.73	101	77	
	12	2.10	100	80	
	32	1.82	100	66	
	69	1.70	96	78	
" 8	58	1.60	114	64	Died.
	2	1.65	123	86	
	6	2.27	128	109	
	3	2.20	128	95	
" 11	21	1.90	105	83	
	22	1.75	100	82	
	52	1.80	127	103	
	95	1.92	122	117	

n this solvent. We did not find out whether the insoluble part was active. The procedure employed was quite similar to the one given for Preparation A (second adsorption) and crystalline insulin.

40 mg. of the insulin preparation which had been purified by soelectric precipitation and two adsorptions on charcoal were

mixed well with 20 cc. of M/15 disodium hydrogen phosphate. 15 minutes after standing the solution was centrifuged and the clear supernatant fluid poured off and shaken with 20 mg. of supranorit (preparation of Dingemans) for half an hour. The charcoal powder was extracted three times with 1 cc. of 90 per cent phenol. The insulin preparation thus obtained was standardized against crystalline insulin the same day, immediately after it was obtained. According to Dingemans this preparation should be much more active than crystalline insulin. Tables IV and V clearly show, however, that we were not able at any time to secure a preparation which was more active than crystalline insulin; the activity was about the same as that of crystalline insulin. The experiment just outlined has been carried out independently by two investigators in this laboratory, with the same results.

Dingemans found that this final product is rather unstable and loses most of its activity whether kept in solution or in dry form. We could confirm this finding of Dingemans. As can be seen from Experiments 5 and 6 in Table I the Dutch insulin preparation has lost a great part of its activity when let stand in solution overnight. Crystalline insulin will lose no appreciable amount of its activity under these conditions. In the third series of these experiments when we used an insulin preparation which had been kept in a desiccator for several days, the result was the same.

We were interested to see if crystalline beef insulin would become also more unstable by adsorption on charcoal. Crystalline insulin was therefore adsorbed on charcoal, and the obtained product kept in M/15 disodium hydrogen phosphate solution overnight, and was then compared with crystalline insulin. As Experiments 7 and 8 in Table I indicate, no loss in activity could be observed.

IV. Adsorption of Pig Insulin on Charcoal.

We have been informed that the Dutch insulin preparation (organon) contains about 70 per cent of pig insulin. We thought that perhaps pig insulin might give a more active preparation than crystalline beef insulin. For this reason we studied the adsorption of pig insulin on charcoal. The preparation of pig

insulin,¹ from which we started, was evaluated and found to be about as active as crystalline insulin. The adsorption on charcoal was carried out similarly to that previously outlined. Experiments 9 and 10 (Table I) show that the adsorbed preparation was not more active than crystalline beef insulin but had about the same activity.

We also found that the adsorbed pig insulin does not lose any appreciable amount of its activity by letting it stand in $m/15$ disodium hydrogen phosphate solution overnight, as Experiments 11 and 12 demonstrate.

CONCLUSIONS.

The experiments outlined in this paper clearly indicate that we have not been successful in obtaining a preparation more active than crystalline beef insulin. We therefore cannot substantiate the reported finding by Dingemans of an insulin preparation which according to her should contain about 150 international units per mg. We are not in a position to explain the results of Dingemans. Unfortunately, Dingemans did not compare her highly active preparation with a standard preparation, which in our minds is quite important in determining the absolute value for the activity of an insulin preparation. As can be seen in Experiments 9 and 11 (Table I) and in Table II, from the figures obtained with crystalline insulin, we have obtained sometimes quite a lowering of blood sugar with a comparatively small dosage of crystalline insulin. According to the method of evaluation employed by Dingemans, these figures would give a much higher value for the activity of crystalline insulin than is obtained on a comparative basis.

SUMMARY.

Starting with various insulin preparations—pyridine precipitate, crystalline insulin, and pig insulin—and submitting them to the adsorption on charcoal according to Dingemans, using the same grade as employed by Dingemans, we have completely failed in obtaining a product more active than crystalline insulin.

¹ The material was kindly prepared for us by E. R. Squibb and Sons, New Brunswick, New Jersey, and we wish to express our best thanks to them.

In repeating the work of Dingemane using the same insulin preparation and charcoal as employed by Dingemane, we were not able to secure a preparation more active than crystalline insulin. We found in agreement with Dingemane that the final preparation obtained from organon is rather unstable. The results of our experiments do not substantiate the claim of Dingemane to be able to obtain an insulin preparation which is more active than crystalline insulin.

Addendum.—When this paper was in press we received an answer from Doctor Dingemane to whom we had sent a copy of the manuscript. According to this personal communication one has to use a certain amount of supranorit for a given quantity of insulin and with different insulin preparations one always has to find out first the exact amount of supranorit to be used in order to obtain positive results. With the amount of supranorit employed in her recent experiment with crystalline insulin Doctor Dingemane was not able to secure a more active preparation than the crystalline insulin itself, but she thinks that this negative result may be due to the fact that the exact amount of charcoal necessary for the success of the experiment had not first been ascertained. Doctor Dingemane also informed us that she has lately compared the highly active preparations obtained by her with crystalline insulin and again reports that her preparations have a higher activity. It seems to us that the experimental conditions which should give positive results have to be put on a much sounder basis before her results can generally be accepted. We think, however, that one has to maintain an open mind on the possibility of preparing from pancreatic extract a product more active than crystalline insulin. Although our results in repeating the work of Dingemane as closely as possible have been negative in the sense that we were not able to secure a preparation more active than crystalline insulin, we present our findings with the hope of inducing other laboratories to repeat the work. Doctor Dingemane also informs us that workers in other laboratories have been unable to repeat her work. We should like to express here again our great appreciation for the cooperation given us by Doctor Dingemane.

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