AN *IN VIVO* TEST FOR THE HYPERGLYCÆMIC GLYCOGENOLYTIC FACTOR IN INSULIN

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It has been reported that most brands of American insulin contain a hyperglycæmic glycogenolytic factor. The literature on this subject has been adequately reviewed by Pincus[1]. This insulin contaminant is a protein which is extractable from the pancreas and is also said to be present in dog gastric and duodenal mucosa[2]. Some observations made suggest that the factor may be a hormone secreted by the α cells of the pancreas or by the argentophil cells which are present in gastro-intestinal mucosa, but absolute evidence of this fact is lacking[3]. Some methods of insulin manufacture fail to remove this factor since it survives the many purification processes involved. Our problem was to assess the factor present in certain samples of British insulin or to endeavour to prove their freedom from it.

Various *in vitro* and *in vivo* tests for this factor have been described in the literature. The *in vitro* method adopted by many workers is one described by Sutherland and Cori[4] which is a liver slice technique based on the findings of Shipley and Hümel[5]. It has been shown that the property of stimulating glycogenolysis in isolated liver slices is due to the factor contained in the insulin. Most of the *in vivo* work has been carried out by intravenous injection and infusion techniques. It has been claimed that the subcutaneous, intramuscular and intraperitoneal injection of this factor fails to cause hyperglycæmia[3]. The experiments reported in the literature have been carried out on unmodified insulin and on insulin inactivated by the method of Sutherland and Cori[4] using several species of animal with and without anæsthetic. The doses of insulin given have tended to be large and in many cases have been massive.

This paper is concerned with the description of an *in vivo* method of test on rabbits, in which the animal is sensitive to the factor present in a moderate dose of insulin without previous inactivation of the hypoglycæmic activity. The use of inactivated insulin is open to the criticism that the degradation products of inactivation may cause hyperglycæmia. Experimental work has been carried out to determine the most suitable assay procedure. Such questions as dose level, diet in relation to response, route of administration and time of blood sugar observations after injection, have been studied. It has been found that the variability of blood sugar response between rabbits necessitates the application of a cross-over test technique.

Experimental

Three test substances have been used in these experiments:

A. American insulin which is reputed to contain the hyperglycæmic glycogenolytic factor.

B. British insulin manufactured in these laboratories.

C. Danish insulin which is reputed to be free from, or to contain very little activity due to this factor.

Series I. Preliminary tests were carried out by the well-known cross-over technique in which on the first day half of the animals received the test substance and the other half received the standard. The second day the procedure was reversed. The reputed positive insulin A was used for a standard. At least 3 days' interval was given between each successive day of test. The same 6 rabbits (maintained on laboratory diet of oats, hay and cabbage) were used throughout the series and they were starved for 21 hours before use. The dosage of insulin was 0·2 I.U./kg. given as a 1·0 I.U./ml. solution in acid-glycerin-cresol water, *p*H 3·2 by intravenous injection into the marginal ear vein. 2 blood sugar estimations were made on different blood samples withdrawn immediately before injection and the mean of these was taken as the initial blood sugar level. Following injection the animals were bled from the ear vein at 2-minute intervals for the first 10 minutes and thereafter less frequently for 1 hour. Blood sugar determinations were made by Benedict's method[8].

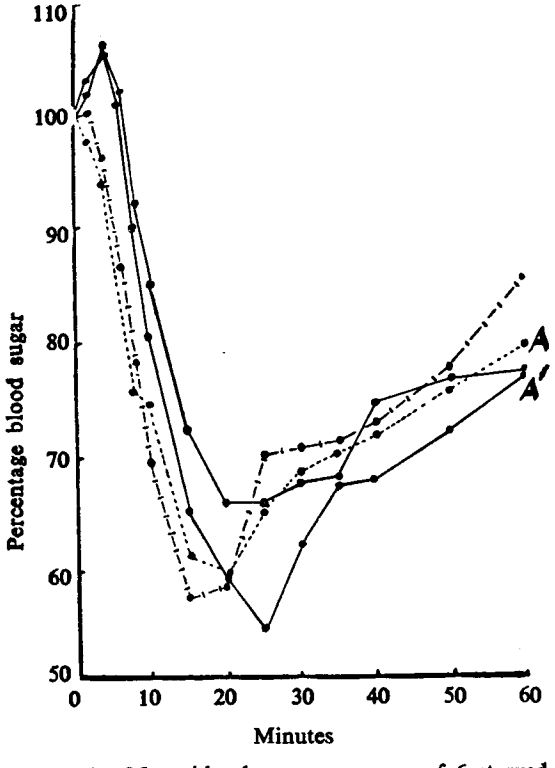

FIG. 1. Mean blood sugar responses of 6 starved rabbits. Actual blood sugar values are expressed as percentages of initial level. Dose, 0·2 I.U./kg.

———— Insulin A.
- - - - - - - „ B.
—|—|—|—|— „ C.

The mean blood sugar responses of the 6 animals which received each of the 3 test substances are shown in Figure 1. The actual blood sugar values have been expressed as a percentage of the initial level.

Using the intravenous technique described above it was possible to demonstrate the presence of the factor in insulin A in two separate tests, which had an interval of 3 months between them. Hyperglycæmia was

not produced following the injection of insulin B, the onset of hypoglycæmia commenced 2 minutes after the injection. Insulin C also failed to produce hyperglycæmia but the hypoglycæmia was not immediate.

Tables I and II show a statistical analysis of the mean blood sugar responses obtained during the first 8 minutes after injection. The variance ratio " F " test has been applied to ensure that the variations between rabbits within different insulins are homogeneous. The " t " test has been applied to examine the difference between means.

TABLE I

Minutes after injection	Mean blood sugar as percentage of initial value				Sum of squares			
	A	A^1	B	C	A	A^1	B	C
2	103·1	102·3	97·8	100·3	227	286	382	68
4	105·6	106·1	94·0	96·1	142	302	138	340
6	102·3	101·1	85·1	86·5	327	203	410	571
8	92·2	90·2	75·7	78·1	249	92	362	636

TABLE II

Comparison	Time minutes	F	$n_2 \backslash n_1$ D.F.	p		t	D.F.	p	
B *v.* A...	2	1·684	5\5	>0·2		1·177	10	>0·2	<0·3
	4	1·029	,,	>0·2		3·755	10	>0·001	<0·01*
	6	1·255	,,	>0·2		3·470	10	>0·001	<0·01*
	8	1·454	,,	>0·2		3·66	10	>0·001	<0·01*
B *v.* A^1...	2	1·333	,,	>0·2		0·953	10	>0·3	<0·4
	4	2·185	,,	>0·2		3·19	10	>0·001	<0·01*
	6	1·639	4\5	>0·2		3·19	9	>0·01	<0·02*
	8	3·930	5\5	>0·05	<0·1	3·73	10	>0·001	<0·01*
B *v.* C...	2	5·610	,,	>0·01	<0·05	0·632	10	>0·5	<0·6
	4	2·458	,,	>0·1	<0·2	0·544	10	>0·5	<0·6
	6	1·390	,,	>0·2		0·25	10	>0·8	<0·9
	8	1·758	,,	>0·2		0·416	10	>0·6	<0·7
A^1 *v.* C...	2	4·210	,,	>0·05	<0·10	0·595	10	>0·5	<0·6
	4	1·125	,,	>0·2		2·17	10	>0·05	<0·1
	6	2·248	4\5	>0·2		4·256	9	>0·001	<0·01*
	8	6·900	5\5	>0·01	<0·05	2·45	10	>0·02	<0·05*

* Significant difference.

From the analysis it is concluded that at the P = 0·05 significance level, in the two tests on A and A^1 insulin, the blood sugar responses are significantly different from the responses following the administration of B insulin at 4, 6 and 8 minutes, but not at 2 minutes. Also responses to A^1 insulin are not significantly different from those to C insulin 2 minutes after injection, they are nearly significant at 4 minutes and at 6 and 8 minutes the difference is highly significant. No significant differences occur between the blood sugar responses to B and C insulin during the first 8 minutes.

Series II. A cross-over test of 3 × 3 latin square design was next undertaken. This enabled a direct comparison of the 3 substances to be made simultaneously. The test was carried out on a new group of 6 rabbits. An interval of 1 week was allowed between successive days of the test. The animals were given ordinary laboratory diet and starved for 21 hours before use. Injections were administered intravenously and the dose given was 0·4 I.U./kg. This was twice the dose used in Series I.

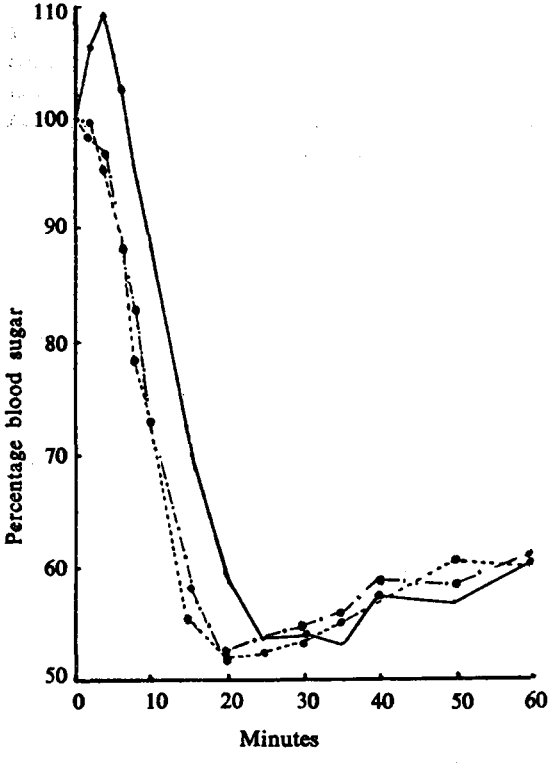

FIG. 2. Mean blood sugar responses of 6 starved rabbits. Actual blood sugar values are expressed as percentages of initial level. Dose, 0·4 I.U./kg.

———— Insulin A.
- - - - - - - „ B.
– · – · – · – „ C.

The results are shown in Figure 2, with a statistical analysis in Tables III and IV.

This experiment showed that A insulin, administered intravenously, produced a hyperglycæmic response in the starved rabbit which lasted for 6 minutes, whereas B and C insulin under the same conditions showed no hyperglycæmia. Therefore, the experiment confirmed the earlier cross-over tests.

Inter-rabbit variation in response to the injection of A insulin was much

TABLE III

Minutes after Injection	Mean blood sugar as percentage of initial value			Sum of Squares		
	A	B	C	A	B	C
2...	106·3	99·6	98·1	202	141	17
4...	109·2	95·2	96·2	1114	68	51
6...	102·6	90·0	88·2	401	73	50
8...	95·8	78·5	83·1	749	109	115

greater than that shown in response to the injection of C or B insulin. Table IV shows that as a result of this the F values in comparisons of B *v.* A and C *v.* A are rather high in some cases. This should be taken into consideration when quoting significant differences between A and the other two insulins in this test on starved animals. However, where the probability that the two series are homogeneous is less than 1 per cent. the "t" test for significant difference between means has not been applied.

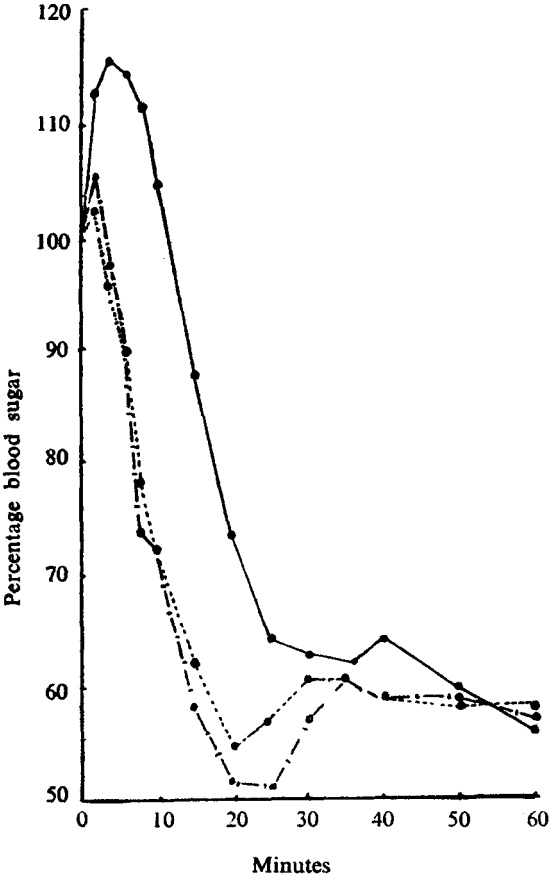

FIG. 3. Mean blood sugar responses of 6 unstarved rabbits. Actual blood sugar values are expressed as percentages of initial level. Dose, 2·0 I.U./kg.

———— Insulin A.
- - - - - - - „ B.
-·-·-·-·- „ C.

Series III. In experiments on crude pancreatic extracts it was found that animals receiving glucose "per os" and later animals with hepatic glycogen reserves increased by the addition of carrots to their diet, were more sensitive and gave consistent hyperglycæmic responses. This latter finding is in agreement with Weisberg *et al.*[3].

A second cross-over test of 3 × 3 latin square design was carried out on another series of 6 rabbits maintained on normal laboratory diet plus carrots *ad lib.* throughout the experiment. This was designed with the object of obtaining more significant differences between the three insulin preparations. The injections were administered intravenously and the dose given was increased to 2·0 I.U./kg.; this was 5 times the dose given to the starved animals in Series II.

TABLE IV

Comparison	Time minutes	F	n_2 \ n_1 D.F.	p		t	D.F.	p	
B *v.* A...	2	1·433	5\5	>0·2		1·981	10	>0·05	<0·1
	4	16·38	,,	>0·001	<0·01	—	—		—
	6	5·493	,,	>0·01	<0·05	3·170	10	sl.	<0·01*
	8	6·872	,,	>0·01	<0·05	3·240	10	sl.	<0·01*
B *v.* C...	2	8·294	,,	>0·01	<0·05	0·641	10	>0·5	<0·6
	4	1·333	,,	>0·2		0·517	10	>0·6	<0·7
	6	1·460	,,	>0·2		0·914	10	>0·3	<0·4
	8	1·055	,,	>0·2		1·702	10	>0·1	<0·2
C *v.* A...	2	11·88	,,	>0·001	<0·01	—	—		—
	4	21·84	,,	>0·001	<0·01	—	—		—
	6	8·02	,,	>0·01	<0·05	3·727	10	>0·001	<0·01*
	8	6·513	,,	>0·01	<0·05	2·363	10	>0·02	<0·05*

* Significant difference.

The results are shown in Figure 3 and a statistical analysis is given in Tables V and VI.

TABLE V

Minutes after injection	Mean blood sugar as percentage of initial value			Sum of squares		
	A	B	C	A	B	C
2...	112·8	103·2	105·3	384	152	70
4...	115·7	97·5	95·3	108	25	46
6...	114·5	89·7	90·0	153	154	278
8...	111·5	78·2	74·0	831	93	423
10	104·8	72·2	72·0	1078	93	68

TABLE VI

Comparison	Time minutes	F	n_2 \ n_1 D.F.	p		t	D.F.	p	
B *v.* A...	2	2·526	5\5	>0·1	<0·2	2·271	10	>0·02	<0·05*
	4	4·32	,,	>0·05	<0·1	8·643	10		<0·001*
	6	1·0	,,	>0·2		7·752	10		<0·001*
	8	8·935	,,	>0·01	<0·05	5·766	10		<0·001*
	10	11·59	,,	>0·001	<0·01	—	—		—
B *v.* C...	2	2·171	4\5	>0·2		0·698	9	>0·5	<0·6
	4	1·840	5\4	>0·2		1·293	9	>0·2	<0·3
	6	1·805	,,	>0·2		0·072	9	>0·9	
	8	4·548	,,	>0·05	<0·1	0·916	9	>0·3	<0·4
	10	1·368	4\5	>0·2		0·078	9	>0·9	
C *v.* A...	2	5·486	,,	>0·05	<0·1	0·744	9	>0·1	<0·2
	4	2·347	,,	>0·2		8·145	9		<0·001*
	6	1·817	5\4	>0·2		5·654	9		<0·001*
	8	1·964	4\5	>0·2		5·247	9		<0·001*
	10	15·85	,,	>0·001	sl.<0·01	—	—		—

* Significant difference.

The very marked hyperglycæmic response obtained with insulin A in this experiment has confirmed that animals with increased liver glycogen are more sensitive to the hyperglycæmic glycogenolytic factor than starved animals, also they are less sensitive to insulin; a dose 5 times that given

to starved animals produced approximately the same hyperglycæmic effect. Statistical analysis has shown that the difference in response between insulin A and B and between insulin A and C is significant at the P=0·05 level during the first 8 minutes. It has also been shown that there is no significant difference between the response to insulin B and C.

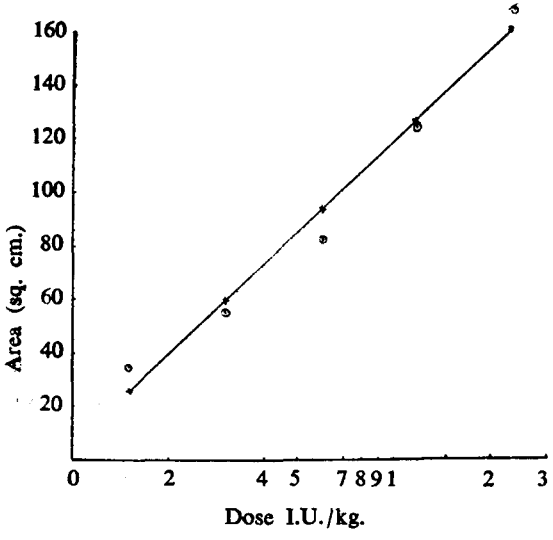

FIG. 4. Regression of area under the hyperglycæmic curve on the logarithm of the dose of Insulin A.

⊙ Observed value, mean of 6 rabbits
× Calculated value
Equation Y = 111·4 x + 5·63

Series IV. Having shown a significant hyperglycæmia with doses of up to 2·0 I.U./kg. of insulin A, it was thought to be of interest to explore the effect of higher dose levels using the technique described. Preliminary trials showed promising results in regard to a progressive increase of response to dose. Therefore a cross-over test was carried out from which the mean response of 6 animals was obtained for doses of 1·5, 3·0, 6·0, 12·0 and 24·0 I.U./kg. The test was performed in two parts. In the first experiment a 3 × 3 latin square design was used employing the doses 1·5, 3·0 and 6·0 I.U./kg. This was extended by a 2 × 2 latin

TABLE VII

AREA UNDER HYPERGLYCÆMIC CURVE

Dose I.U./kg.	1·5	3·0	6·0	12·0	24·0
Log dose	0·1761	0·4771	0·7782	1·0792	1·3802
Rabbit :—					
E.13	13·68	23·82	14·87	41·44	106·50
E.14	55·03	49·14	73·75	38·98	73·72
E.15	22·35	31·62	31·55	147·34	238·24
E.16	33·48	104·35	76·53	214·11	119·93
E.17	16·85	84·96	115·30	172·27	409·10
E.18	64·33	34·10	176·77	128·62	56·97
Total	205·72	327·99	488·77	742·76	1004·46
Mean	34·29	54·67	81·46	123·79	167·41

square test on the same animals given 12·0 and 24·0 I.U./kg. In order to take into account the duration as well as the magnitude of the response, the area under the hyperglycæmic response curve obtained from each animal was measured. The results are given in Table VII. Figure 4 shows the mean areas plotted against log dose. The best fitting straight line was calculated and drawn through the points. A statistical analysis of the results is shown in Table VIII.

TABLE VIII

VARIANCE ANALYSIS

Nature of variation	D.F.	Sum of squares	Mean Square	F	P
Regression	1	67,479	67,479	12·04	>0·001 <0·01
Deviations from regression ...	3	1,716	572	0·102	>0·20
Between doses	4	69,195			
Within doses	25	140,166	5,606·64		
Total	29	209,361	—		

Therefore the regression between the area under the blood sugar curve and the logarithm of the dose is highly significant and there is no reason to doubt the hypothesis of linearity.

DISCUSSION

A dose of 2·0 I.U./kg. of insulin B or C given to fed animals failed to give a hyperglycæmic response, whereas a dose of 0·2 I.U./kg. of insulin A produced a significant hyperglycæmia even in the less sensitive starved animal. Sutherland *et al.*[6] have estimated that insulin of type A may contain 5 to 10 per cent. of hyperglycæmic glycogenolytic factor. Therefore, a dose of 0·2 I.U./kg. of insulin A would equal approximately 0·0009 mg./kg. of the factor assuming a potency of 22·0 I.U./mg. for insulin A. It appears from our results that insulin B and C are either free from, or contain less than 1·0 per cent. of the factor.

The foregoing experiments have shown that hyperglycæmic activity can be most satisfactorily demonstrated by using a latin square cross-over technique on rabbits with increased hepatic glycogen reserves. Blood sugar determinations at 2 minute intervals are necessary during the first 10 minutes after injection to detect the very transitory hyperglycæmia due to the presence of small quantities of the factor.

In experiments on well-fed non-anæsthetised rabbits given insulin of type A by continuous infusion technique, Weisberg *et al.*[3] state, "The degree of hyperglycæmia bears no consistent relationship to the dose of hyperglycæmic glycogenolytic factor administered." Loube *et al.*[7] obtained very marked hyperglycæmic responses in man and in several species of experimental animal under anæsthesia, by intravenous injection of 0·05 mg./kg. of an extract of insulin which was stated to contain that amount of the factor which would be present in an equal weight of commercial insulin. They found that increasing the amount of extract administered to cats from 0·05 to 0·5 mg./kg. did not increase the

hyperglycæmic effect obtained. It may be noted, however, that the low dose, 0·05 mg./kg. gave over 100 per cent. increase in the blood sugar.

Our own experiments on insulin A indicate that there is a linear relationship between hyperglycæmia as measured by the area under the response curve, and the logarithm of the dose of factor given. This has been shown to be true over the dose range 1·5 to 24·0 I.U./kg. of insulin A. Other groups of animals, not reported here, given doses of insulin A within this range have been found to give results which agree well with the fitted regression line shown in Figure 4.

It is interesting to note that with a considerable increase in dosage of insulin A containing a low percentage of the factor the hyperglycæmic response is not inhibited by the insulin but, on the contrary, the onset of the hypoglycæmia is delayed.

SUMMARY

1. An *in vivo* method has been described for demonstrating the presence of the hyperglycæmic glycogenolytic factor in insulin, in which the insulin can be administered in an unmodified form and significant responses may be obtained with a moderate dose.

2. The results so far obtained using the technique described show that British and Danish insulin do not exhibit activity due to this factor.

3. A linear relationship between the area under the blood sugar curve and the logarithm of the dose of the factor has been established.

We wish to thank the Directors of Allen and Hanburys Ltd., and The British Drug Houses Ltd., for permission to publish this work.

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DISCUSSION

The two papers were presented by DR. F. HARTLEY.

The CHAIRMAN said that the papers were of great interest particularly to insulin manufacturers. It was fortunate that British and Danish insulins appeared to be free from the hyperglycæmic factor whereas the American insulin process might have to be altered.

MR. T. D. WHITTETT (London) referred to dermal reactions to insulin in two patients who had not responded to treatment with antihistaminic drugs. That was rather rare and the two patients were being treated with six times recrystallised insulin from Sweden. He asked whether other workers had experienced any similar lack of response to the action of antihistaminic drugs.

DR. G. FOSTER (Dartford) said he understood that in the work described the insulin had been tested for the hyperglycæmic factor both in animals and clinically. According to some authorities that factor could only be detected clinically when insulin was injected intravenously, and since he understood that particular route of injection was not employed clinically it would be interesting to learn how the clinical test was carried out. The withdrawal of blood from the rabbit's ear at 2-minute intervals would cause stimulation which might be reflected in the glucose content of the rabbit's blood. That being so, he asked whether any control experiments had been carried out.

DR. HARTLEY, in reply, pointed out that the type of reaction referred to by Mr. Whittett had been described by Paley, who emphasised that desensitisation might be required in certain patients and also that antihistamines might not be capable of combating the effect. Further, Paley also examined 6-times recrystallised insulin and found that it was indistinguishable, by partition chromotography, from twice recrystallised insulin. It was not homogeneous. In their experiments the insulins were examined *in vitro* and *in vivo* only, whereas the Americans had examined their products both *in vivo* and clinically by intravenous injection. It was not apparent why larger doses could be given intravenously than intramuscularly without producing convulsive response. The possibility of stimulation of the blood sugar by repeated withdrawal of blood samples had been examined and did not arise with expert handling of the animals. The immediate results were erratic but significant results could be obtained after the first 2 minutes.