THE CONCENTRATION OF ADENOSINETRIPHOSPHATE (ATP) CITRATE AND CALCIUM IN BLOOD DURING INSULIN SHOCK THERAPY

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CONCENTRATION changes of blood constituents other than glucose occurring during hypoglycaemia, and particularly during insulin shock therapy, have been studied by several authors (e.g., Harris, Blalock and Horwitz, 1938; Katzenelbogen et al., 1939) though not always with concordant results. One of the most constant features of insulin hypoglycaemia is a decrease in the level of inorganic phosphate during the phase of blood-sugar fall and a gradual return to normal after the blood-sugar curve has reached its lowest value. This fact which was described soon after the discovery of insulin (see Cori (1931) for a review of the older literature) is generally attributed to the increased phosphate esterification consequent upon the enhancement of glucose utilisation and is in harmony with the current concept that the function of insulin is primarily that of a promotor of glucose phosphorylation.

It seemed of interest to study the level of adenosine triphosphate (ATP) in blood during insulin-shock therapy in view of its intimate connection with glucose phosphorylation. The maintenance of an adequate rate of glucose phosphorylation and that of an adequate level of ATP are mutually interdependent processes, at least in isolated enzyme systems, and a fall of either one or the other might initiate a dangerous vicious circle. Since Olsen and Klein (1947) reported a fall in the concentration of ATP in the brain of hypoglycaemic cats it is important to know how general this phenomenon is. The ATP of blood which is entirely intracellular (Kerr and Daoud, 1935) is metabolically very active with a turn-over rate as high as the ATP of liver (Hevesy, 1947); it may therefore be expected to reflect to some extent the ATP-metabolism of the majority of tissues.

The citric acid content of serum was studied because citric acid may be involved in the hypoglycaemic syndrome for two reasons: as a member of the tricarboxylic acid cycle it is linked to glucose metabolism and it is also intimately connected with calcium metabolism and through it may be influenced by the concentration of inorganic phosphate.

Finally, serum calcium levels were investigated in a small number of cases because of the connections with phosphate and citrate metabolism. Serum calcium levels have previously been studied, but with conflicting results: whereas Accornero and Bini (1937) reported a decrease in hypoglycaemia, Georgi (1936) found increases and several other authors (Beiglböck and Dussik,

1937 ; Harris et al., 1938 ; Katzenelbogen et al., 1939 ; Delay et al., 1945) came to the conclusion that there was no significant change.

METHODS.

The subjects were chosen at random from the patients undergoing insulin shock therapy at this hospital. They received an individually adjusted dose of insulin designed to produce hypoglycaemic coma 3-4 hours after intramuscular injection ; it varied from about 40 units to about 250 units. Four venous blood samples were collected from each subject : (1) before insulin injection ; (2) two hours after injection ; (3) during coma, and (4) one hour after termination of coma. In the series of calcium determinations a fifth sample was collected one hour after insulin injection.

Serum was used for the analysis of citric acid and calcium. For the estimation of ATP 5 ml. blood were drawn up into a syringe and immediately delivered into a graduated, glass stoppered cylinder containing 20 ml. ice cold trichloracetic acid (20 per cent.). This procedure minimises the enzymatic breakdown of ATP after the withdrawal of blood.

Estimation of ATP.—ATP was determined in the trichloracetic acid filtrate by the increase of inorganic P produced by purified myosin which specifically converts ATP into adenosine diphosphate (ADP) and inorganic phosphate. Phosphate liberated by myosin will be called the " myosin-P " fraction. In addition the fraction hydrolysable by *N* HCl in 10 minutes at 100° C. (the " 10′-P " fraction) was determined.

Phosphate estimations were usually done by the method of Berenblum and Chain (1938). Sometimes the less laborious, but less accurate method of Fiske and Subbarow (1925) was used.

A detailed description of the analytical procedure follows. The trichloracetic acid filtrate was measured and 1 ml. *N* HCl was added. The acid solution was extracted four times with 10 ml. portions of peroxide-free, redistilled ether to remove excess trichloracetic acid. The volume of the extracted solution was again read and, after neutralisation, ether was removed by evacuation and gentle warming in a water bath. After the volume had been made up to 20 ml. or else noted down, inorganic and 10′-P fractions were determined in suitable aliquots (1 ml. for inorganic P and 0·5 ml. for 10′-P fraction, when the Berenblum and Chain method was used ; with the Fiske and Subbarow method fourfold quantities are required).

Myosin was prepared from rat muscle essentially according to Bailey (1942) with some modifications. The skinned and eviscerated carcase of a rat was packed in cracked ice for 15 minutes. Sufficient muscle was excised and passed through a cooled Latapie mincer to collect 10 g. of ground tissue. This was immediately suspended in 50 ml. of cold solution of 0·5 *M* KCl+0·03 *N* $NaHCO_3$ and gently stirred for one hour at 0°. The pH was maintained at 7·0-7·5 by adding a pinch of solid $NaHCO_3$ once or twice during the first 10 minutes. The centrifuged extract was clarified by filtration through a layer of paper pulp, diluted with 200 ml. of ice-cold glass-distilled water and brought to pH 6·8 by the addition of *N*/10 HCl. The precipitated myosin was immedi-

ately centrifuged and redissolved in 30 ml. of the KCl—$NaHCO_3$ solution. Precipitation by dilution with 200 ml. water and solution in 30 ml. KCl—$NaHCO_3$ was repeated until the protein had been three times reprecipitated. No acidification is necessary after the second and third dilution. The final precipitate was stirred up in 15 ml. KCl—$NaHCO_3$ solution and the total volume measured. Solid KCl was then added to bring the concentration up to 0·5 *M* whereupon the remaining precipitate was easily dissolved. It is important for the preparation of active extracts to keep the temperature as low as possible, to use glass-distilled water throughout and to avoid delay during the process of reprecipitation. The preparation remained sufficiently active for at least a week when kept at 0°. For use it was diluted with an equal volume of 0·2 *M* glycine—NaCl buffer of pH 9·1.

The estimation of " myosin-P " was carried out in two test tubes each containing a 6 ml. aliquot of the ether-extracted neutral filtrate and in addition 0·2 ml. of 0·1 *M* $CaCl_2$; 1·8 ml. 40% trichloracetic acid was added to one of the tubes, followed by the addition of 2 ml. myosin in glycine buffer to both. After 30 minutes incubation at 37° the same amount of trichloracetic acid was added to the second tube. The " myosin-P " fraction is given by the difference of the P-values.

Estimation of citric acid.—Three ml. serum were added slowly to a mixture of 20 ml. water, 3 ml. 10 per cent. sodium tungstate and 3 ml. 0·67 *N* H_2SO_4. A 20 ml. aliquot of the filtrate was concentrated on a hot-plate to a volume of 2-3 ml. and analysed by the method of Weil-Malherbe and Bone (1949). The petroleum ether extract was shaken with 1 ml. Na_2S solution in a centrifuge tube and, after short centrifugation, an aliquot of the aqueous layer was transferred to the micro cell of the Spekker absorptiometer with the aid of a fine-tipped teat pipette.

Estimation of calcium.—The method of Wang (1935) was used.

Blood sugar was estimated by the method of Nelson (1944).

Tests of statistical significance.—In each group the analytical results represent four or five series of values of which one value in each series is contributed by the same individual. If the means of two series were compared on the basis of the variance of the two series, the values would be treated as random samples and no account would be taken of the pairing of values. The tests of significance were therefore based on the variance of the differences of two paired values x_1 and x_2 and t was calculated by the following formula :—

$$t_{(n-1)} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\dfrac{\Sigma(x_1 - x_2)^2 - \dfrac{(\Sigma x_1 - \Sigma x_2)^2}{n}}{n(n-1)}}}$$

where $\bar{x}_1$ and $\bar{x}_2$ are the means of the two series of values x_1 and x_2 and n the number of differences.

Results.

Blood-sugar values were determined in a large number of cases in this and other series of investigations. The lowest level of blood sugar, varying from

practically 0 to about 20 mg per cent., is usually reached two hours after the injection of insulin. The onset of coma takes place 1-2 hours later when the blood sugar has either remained unchanged or has shown a slight rise. One hour after the termination of coma the blood sugar has reached a more or less normal level again. The detailed communication of these figures has been omitted in view of the wealth of similar data in the literature.

The analyses of inorganic P, myosin-P and 10′-P, contained in Table I, show that there is a marked fall of the inorganic phosphate level two hours after insulin injection reaching an average of about 70 per cent. of the pre-injection value. During coma, i.e. 3-4 hours after the injection, the level has risen again to about 82 per cent. and in the fourth series it has practically returned to normal. The differences between the means of these series are all significant.

The level of ATP, as measured by the myosin-P fraction, remains practically constant two hours after insulin injection. In the two following series the ATP concentration increases to 110 and 116 per cent. above the initial level. Both increases are significant (comparison was made between series 2-3 and 2-4).

This increase is not shown, however, by the figures of the 10′-P fraction; on the contrary, there is a significant decrease in the second series. Later, a return to the pre-injection level is observed.

The last four columns of Table I show the percentage of 10′-P accounted for by ATP, as determined by the myosin method. It is obvious that the mean figures are all significantly below 100 and that ATP does therefore not, as is often assumed, account for the entire 10′-P fraction. In the pre-injection sample ATP accounts on the average for only about 80 per cent. of the total 10′-P fraction, but under the influence of insulin the percentage increases and reaches a value of 90 in the last sample. This increase, too, is significant. The other component or components of the 10′-P fraction have not been identified, but since the ATP fraction increases at the expense of the unknown residual fraction (the total 10′-P fraction remaining constant) it presumably consists largely of ADP.

Table II contains the results of citric acid analyses carried out on 22 cases. In some of these the blood for citric acid analysis was drawn at the same time as the sample for the estimation of ATP. These cases bear the same serial number in Tables I and II. In spite of greater individual variation the differences of the mean values of the four samples are small. Yet there is a significant decrease from the first to the second sample, followed by a significant increase from the second to the fourth sample. It may be concluded that there is a slight fall of the citric acid level during the first phase of hypoglycaemia, amounting to 15 per cent. on the average, and a return to a near-normal level after termination of the coma. In some cases (No. 1, 14 and 20) a further sharp drop occurred in the fourth series.

The serum calcium was investigated in only eight cases (Table III). The fall of the calcium level during hypoglycaemia, though quantitatively small, is highly significant statistically. There was considerable variation of the time at which the lowest calcium value was observed. Sometimes the minimum was reached as early as one hour after insulin injection, in other cases only

during coma. For the evaluation of the significance of the change occurring during the whole period of hypoglycaemia the lowest of the three values obtained in series 2, 3 and 4 was selected and compared with the initial and final value.

Discussion.

Of the concentration changes described the fall of inorganic phosphate is quantitatively the greatest. It is also that which is probably most directly connected with insulin action since an increased rate of phosphorylation is a necessary preliminary to an increased rate of glucose utilisation. It is in agreement with this interpretation that the level of inorganic phosphate tends to rise again as soon as the phase of rapid glucose utilisation is ended and the blood-sugar level has dropped to a stable value. The blood-ATP remains

Table I.—*The Concentration of Phosphate Fractions in the Blood of Patients insulin shock treatment.*

Sample 1.—Withdrawn before insulin injection.
Sample 2.—Withdrawn two hours after insulin injection.
Sample 3.—Withdrawn during coma.
Sample 4.—Withdrawn one hour after termination of coma.

Case No.	Inorganic P (mg./100 ml. blood). Sample				Myosin-P(mg./100 ml. blood). Sample			
	1	2	3	4	1	2	3	4
1	3·19	1·90	2·75	3·80	2·69	2·59	3·07	3·21
2	4·50	3·14	4·21	4·38	2·15	2·20	2·34	1·97
3	4·26	3·28	3·47	4·08	3·01	2·70	2·76	3·26
4	4·72	2·93	3·44	4·86	3·42	2·66	2·99	2·83
5	4·78	3·10	3·80	5·17	2·05	2·57	2·61	2·49
6	3·92	3·27	3·28	4·03	2·13	2·08	2·15	2·24
7	3·65	1·75	2·22	3·47	2·24	2·25	2·71	3·40
8	2·62	1·53	2·32	3·52	2·10	2·68	2·61	2·35
9	4·81	3·71	4·35	5·45	2·25	2·46	2·51	2·75
10	3·73	2·41	3·54	4·44	2·32	2·21	2·68	2·91
11	3·79	3·46	3·52	4·07	2·96	2·34	2·79	3·33
12	4·92	3·20	3·74	3·90	1·97	2·13	2·04	2·30
13	5·11	3·27	3·09	3·82	2·57	2·27	2·74	2·86
14	3·52	2·64	2·64	3·05	2·14	2·30	2·44	3·10
15	5·36	4·34	4·18	4·75	2·53	3·08	3·35	3·61
16	5·80	3·82	4·50	4·08	3·07	3·15	3·65	3·83
17	3·59	2·94	3·39	3·90	3·40	3·03	3·34	2·88
Mean	4·25	2·98	3·44	4·16	2·53	2·52	2·76	2·90
Standard error ..	0·205	0·181	0·099	0·151	0·116	0·080	0·103	0·125
t* ..	10·62	4·64	6·07	..		4·73	3·62	
p ..	< 0·01	< 0·01	< 0·01	..		< 0·01	< 0·01	

* Calculated from the differences of paired values, see "Methods."

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TABLE I—*continued.*

	10′-P(mg/100 ml blood) Sample				2(Myosin-P) × 100 / 10′-P Sample			
	1	2	3	4	1	2	3	4
1 ..	5·40	4·96	6·03	7·01	99·9	104·5	101·6	91·5
2 ..	5·64	5·41	5·01	4·92	76	81·3	93·7	80·2
3 ..	8·34	6·62	6·43	6·90	72·3	81·6	86	94·5
4 ..	7·28	6·37	6·34	6·60	94	83·5	94·5	86
5 ..	7·60	6·60	7·05	7·16	54	78	74	69·5
6 ..	4·58	5·19	5·56	4·61	93·2	80·3	77·3	97·3
7 ..	4·94	4·96	5·46	5·98	91	90·6	99	113
8 ..	6·51	5·37	7·96	4·78	64·5	100	65·6	98·5
9 ..	5·57	5·89	6·25	6·33	80·7	83·5	80·4	87
10 ..	6·21	5·58	6·56	6·84	74·5	79·3	81·6	85·2
11 ..	5·59	5·17	5·74	6·75	106	90·5	97·5	99
12 ..	4·03	4·30	4·06	4·52	98	97	101	102
13 ..	7·08	7·01	6·70	8·05	72·5	64·8	81·8	71·1
14 ..	7·96	5·90	8·84	7·11	53·8	78	55	87
15 ..	7·09	7·18	7·80	7·75	71·4	87	86	93
16 ..	9·0	7·88	8·95	8·74	68·2	80·0	81·5	87·7
17 ..	7·77	8·10	6·61	6·61	87·5	75	101	87
Mean ..	6·50	6·03	6·56	6·50	79·8	84·5	85·8	90·0
Standard error	0·342	0·261	0·313	0·295	3·80	2·37	3·21	2·61
t* ..		2.55	2·06	2·163	..	3·086		
p ..		0·025	0·06	0·05	..	< 0·01		

* Calculated from the differences of paired values, see "Methods."

constant during this phase indicating a balance between the rates of phosphate transfer and rephosphorylation. The subsequent rise of ATP concentration seems to be at the expense of the ADP-fraction. It indicates a preponderance of the synthetic reaction and may be attributed to the accumulation of organic phosphate esters.

Studies with P_{32} have demonstrated that insulin increases the rate of ATP turnover in muscle (Sacks, 1945; Goranson et al., 1948). In liver, insulin raises also the concentration of ATP (Kaplan and Greenberg, 1944). On the other hand, there is a decrease of ATP and an increase of ADP in the brain of hypoglycaemic cats (Olsen and Klein, 1947), which presumably results from the exhaustion of carbohydrate fuel and the consequent depression of metabolism in this tissue. The high level of ATP stores in liver, blood and other tissues may contribute to the rekindling of brain metabolism during recovery from hypoglycaemic coma.

The slight fall of serum calcium may also be connected with the lowering of the inorganic phosphate level and may be an expression of the tendency to keep the Ca : P ratio constant.

TABLE II.—*Concentration of Citric Acid in Serum of Patients during Insulin Shock Treatment.*

Sample 1.—Withdrawn before insulin injection.
Sample 2.—Withdrawn two hours after insulin injection.
Sample 3.—Withdrawn during coma.
Sample 4.—Withdrawn one hour after termination of coma.

Case No.	Citric Acid (mg/100 ml serum). Sample			
	1	2	3	4
1	2·81	2·25	2·75	2·07
2	1·18	2·14	2·05	2·37
5	1·85	1·51	1·31	1·31
6	2·30	1·84	1·98	2·03
7	1·91	1·41	1·63	1·83
8	1·01	0·71	0·91	1·13
9	1·74	1·39	2·00	1·63
10	2·03	1·93	1·84	1·69
11	1·11	1·09	1·31	1·35
12	2·69	2·17	2·17	2·24
13	1·86	1·45	1·40	1·47
14	1·93	1·65	2·07	1·21
15	1·21	1·45	1·19	1·59
16	1·88	1·45	1·80	2·08
17	2·22	2·41	1·98	2·67
18	2·52	1·79	2·31	2·33
19	1·92	1·49	1·01	1·92
20	2·51	1·91	1·81	1·26
21	2·99	2·46	2·22	3·02
22	1·24	0·72	1·02	1·21
23	2·00	1·48	1·91	2·25
24	2·15	1·99	1·72	2·25
Mean	1·962	1·668	1·745	1·860
Standard error ..	0·119	0·102	0·101	0·111
t*		3·704	1·123	2·52
p		< 0·01	0·30	0·02

* Calculated from the differences of paired values, see "Methods."

The fall of serum citrate may be considered in relation to the virtual disappearance of blood sugar. It may be argued that the failure of glucose supply will eventually lead to a lowering of the level of the metabolic stream fed by it which will affect all derivatives. However, the disappearance of circulating glucose is in the first place due to an opening of the sluice gates and the filling of the stream to capacity. Thus, one would expect an increase rather than a decrease in the level of oxidative metabolites, at least in the first phase of hypoglycaemia while glucose is still being absorbed by the tissues.

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TABLE III.—*Concentration of Calcium in Serum of Patients during Insulin Shock Treatment.*

Sample 1.—Withdrawn before insulin injection.
Sample 2.—Withdrawn one hour after insulin injection.
Sample 3.—Withdrawn two hours after insulin injection.
Sample 4.—Withdrawn during coma.
Sample 5.—Withdrawn one hour after termination of coma.

Case No.	Calcium (mg./100 ml. serum). Sample				
	1	2	3	4	5
25	9·8	9·9	9·6	9·9	10·3
26	11·9	11·1	11·1	10·3	12·4
27	11·6	10·3	10·4	10·4	10·8
28	9·4	9·2	8·9	9·0	9·8
29	10·2	9·7	9·7	9·4	10·2
30	10·6	9·8	10·0	10·2	11·0
31	10·0	9·6	9·4	10·2	11·0
32	11·2	10·3	9·4	10·0	11·3
Mean	10·59	9·99	9·81	9·92	10·85
Standard error ..	0·317	0·204	0·242	0·172	0·282
t*		4·79			5·78
p		< 0·01			< 0·01

* Calculated from the differences of paired values, see "Methods."

The fact that the fall of citrate occurs early in hypoglycaemia suggests that the explanation must be sought elsewhere. It is possible, e.g., that this change is linked with the changes of calcium and phosphate concentration. The connections of citric acid and calcium metabolism have long been recognised and Alwall (1945) has shown that the concentrations of both substances in serum often move in the same direction.

Natelson et al. (1948), in experiments which came to the writer's notice after the present work had been completed, found a decrease of serum citric-acid levels after glucose intake and also, in a series of six cases, after insulin injection. In both cases the drop amounted to about 30 per cent. Although this effect is larger than that observed in the writer's cases the results are in general agreement.

SUMMARY.

The blood content of adenosine triphosphate (ATP) and the serum content of citrate and calcium has been studied at various stages before, during and after hypoglycaemic coma and the following observations are recorded :—

(1) In accordance with previous investigators it is found that the curve of inorganic phosphate has fallen by about 30 per cent. two hours after the injection of insulin, but then again moves upwards and has reached normal levels one hour after the termination of coma.

(2) The level of ATP is unchanged two hours after insulin injection, but shows a slight increase later.

(3) ATP accounts for 80-90 per cent. of the "acid-labile" phosphate fraction. As the concentration of ATP increases, that of the unknown residual component decreases. It is suggested that the residue unaccounted for by ATP may consist partly or wholly of adenosine diphosphate.

(4) There is a slight fall of serum citrate during the first phase of hypoglycaemia amounting to about 15 per cent. on the average and a return to normal after termination of coma.

(5) There is a fall of serum calcium during hypoglycaemia which is quantitatively small, but statistically significant.

(6) The changes are discussed and it is suggested that the drop in citrate concentration may be connected with the changes in phosphate and calcium metabolism rather than with changing levels of other glucose metabolites.

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