PARAMETERS IN DETERMINING THE SPECIFIC ACTIVITY OF THE SUCCINATE DEHYDRO- GENASE-COENZYME Q₁₀ REDUCTASE IN MITOCHONDRIA OF LEUCOCYTES

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(Received June 5, 1974)

A study of the parameters has been made of the assay of the specific activities of the succinate dehydrogenase-coenzyme Q₁₀ reductase in mitochondrial preparations from leucocytes to reveal saturation or a deficiency of coenzyme Q₁₀ at its site. These parameters include the key steps from the drawing of blood through the differential determinations for this enzyme system. The variations in the specific activities of the CoQ₁₀-enzyme, both in the absence and in the presence of coenzyme Q₃, was about 10% (mean value). The variations in the final and important criterion, *i.e.*, the deficiency of CoQ₁₀-enzyme activity was about 7%. The variations were not significantly greater, when compared as groups, for duplicate blood samples drawn immediately and sequentially from the same patient.

The data from blood samples collected from the same patient over weeks and months of time showed variations in the specific activities of this CoQ₁₀-enzyme which are believed to be primarily due to changes in the metabolism and/or nutrition of the patient.

Initial data point to at least some correlation of the deficiencies of CoQ₁₀-enzyme activities in leucocytes and skeletal muscle of the same patient. In the future, primary assays for human deficiencies of coenzyme Q₁₀ might be based on leucocytes with subsequent assays of other tissues as specifically justified.

The widely recognized importance of the respiratory chain in bioenergetics connotes a share of importance to the indispensable functionality of coenzyme Q₁₀ in the chain, and this basic knowledge stimulates interest in the potential therapeutic use of coenzyme Q in medicine. Coenzyme Q has the structural and biochemical characteristics of a vitamin, and is a vitamin on the basis of an updated de-

definition of vitamins which recognizes modern knowledge of biosynthesis. On this basis, coenzyme Q should show vitamin activity in experimental animal systems, and it has exhibited such activity in the rabbit, monkey, rat, chick, turkey, and hamster, as summarized by FOLKERS (1).

Coenzyme Q_{10} is indispensable to mammalian life including that of man, and it is reasonable to suppose that human deficiencies of coenzyme Q_{10} can exist; if so, some corresponding ill health should exist. The mildness or severity of such ill health could correlate with the degree of the deficiency of coenzyme Q_{10} . Apparent deficiencies of coenzyme Q_{10} in human disease have been reported by LITTARRU *et al.* (2, 4, 5) and NAKAMURA *et al.* (3) in cardiac disease and periodontal disease, respectively.

A methodology to detect and measure human deficiencies of coenzyme Q_{10} evolved from a pioneer contribution by BRIN (6) in 1960 when he described an enzyme assay based on transketolase to determine thiamine deficiencies. The principle of the enzyme assay was extended to coenzyme Q by NAKAMURA *et al.* (7). The principle of this vitamin assay, as extended by several groups of investigators to several vitamins, was defined by FOLKERS (1) as follows:

—"The specific activity of a coenzyme-apoenzyme system is differentially assayed in the absence and in the presence of added coenzyme. A significant increase in the specific activity of the enzyme system in the presence of added coenzyme measures a deficiency of the coenzyme at the site or of the vitamin in the tissue."

In such an assay for the detection and measurement of deficiencies of coenzyme Q_{10} in human tissue, the succinate dehydrogenase-coenzyme Q_{10} reductase has been differentially assayed in the absence and in the presence of added coenzyme Q_3 . Increasing specific activities in the presence of coenzyme Q_3 which are above 25% are of increasing significance on the deficiency aspects of the clinical situation.

The apparent success of this differential enzyme assay to study human deficiencies of coenzyme Q has made it desirable to study in greater detail the parameters of the assay, particularly on reproducibility and reliability. Repeated determinations of specific activities of mitochondrial preparations from leucocytes of different blood samples from patients, and tests of possible correlation of deficiencies in leucocytes with that of other tissues, have been studied.

METHODS

Blood samples were taken from 51 patients with cardiovascular diseases, which were selected at random, for duplicate or other determinations of the specific activities of the succinate dehydrogenase-coenzyme Q_{10} reductase of the mitochondria from the preparations of leucocytes. These individuals were in the outpatient clinic of the Parkland Memorial Hospital, Dallas.

Ten ml of blood were drawn from the cubital vein of 15 patients, and the mitochondrial preparations were made as described by NAKAMURA *et al.* (7). Duplicate determinations on each of the 15 mitochondrial preparations were performed to appraise variation of the enzymic steps.

Twenty ml of blood were similarly obtained from an additional 15 patients. Ten ml of blood from each patient were drawn into each of two vacutainers, and the leucocyte layers were prepared from both tubes. After making the mitochondrial preparations from both leucocyte layers, the preparations were assayed to evaluate all the isolation and enzymic steps.

Two separate blood samples were obtained from another 21 patients at different times, and the specific activities of the paired blood samples for each patient were determined to provide data on differences due to the times of withdrawal of blood. The period of time between the withdrawal of the paired blood samples varied from 3 to 12 weeks; the mean period was 5.6 weeks.

The vacutainers (127×16 mm) contained 286 USP units of sodium heparin (Becton-Dickinson and Co., Rutherford, New Jersey). Each tube of blood was gently shaken and then centrifuged at $1,000\times g$ for 10 min. The plasma was discarded, and the buffer "layer" and the upper layer of erythrocytes were collected. This leucocyte-rich layer was used for the preparation of mitochondria as described by NAKAMURA *et al.* (7).

The specific activities of the succinate dehydrogenase-coenzyme Q_{10} reductase were determined by the reaction of ZIEGLER and RIESKE (8) with a Beckman Acta C III Spectrophotometer and cuvettes having a light path of 1 cm. Additional details are as follows.

The reaction mixture contained 0.1 ml of 1 M potassium succinate, pH 7.0; 0.1 ml of 1 M potassium phosphate buffer, pH 7.0; 0.015 ml of 0.1% 2, 6-dichloroindophenol (DCIP) with or without 0.01 ml of coenzyme Q_3 solution (1 μ mole/ml in ethanol). Enough water was added to bring the volume to 1.0 ml. After equilibration at 38°C, the reaction was started by the addition of the enzyme solution. Absorbance readings at 600 nm were automatically recorded. In the absence of either enzyme or succinate, the dye was not reduced. Protein was determined by the method of LOWRY *et al.* (9) with recrystallized bovine albumin as a standard. The specific activity (S.A.) with and without CoQ_3 is expressed as nmole of DCIP reduced/min/mg of mitochondrial protein. The activation coefficients (A.C.) and % deficiencies of the activity of the enzyme were calculated as follows:

$$A.C. = \frac{S.A. \text{ with } CoQ_3 - S.A.}{S.A.} \times 100$$

$$\% \text{ Deficiency} = \frac{S.A. \text{ with } CoQ_3 - S.A.}{S.A. \text{ with } CoQ_3} \times 100$$

RESULTS

Duplicate assays on the mitochondrial preparations.

The data on the duplicate assays to determine the specific activities of the succinate dehydrogenase-coenzyme Q_{10} reductase on the same mitochondrial preparations from the same blood samples are in Table 1. The mean value of the variation in specific activity of the duplicate assays was 9.8 ± 2.22 for 15 blood samples, and the value was $9.5 \pm 1.79\%$ for the specific activity in the presence of coenzyme Q_3 . The mean variation in the activation coefficient was $13.1 \pm 2.97\%$, which corresponds to a variation of $6.7 \pm 1.64\%$ for the deficiency of CoQ_{10} -enzyme activity.

1. Duplicate assays of the succinate dehydrogenase coenzyme Q_{10} reductase on the same mitochondria

Patient No.	Specific activity		Specific activity with CoQ_3		Activation coefficient		Percent deficiency	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
1	1.36	1.13	1.36	1.81	0	60	0	38
2	1.79	1.79	2.37	3.56	32	98	24	50
3	1.45	1.26	1.99	1.81	37	44	27	29
4	1.46	1.60	2.00	2.13	37	33	27	25
5	1.00	1.29	1.58	2.14	58	66	37	40
6	1.16	1.16	1.58	1.89	36	63	27	39
7	1.17	1.95	1.95	1.82	67	0	40	0
8	1.41	1.72	2.28	2.20	28	61	38	22
9	1.06	1.22	1.85	1.62	33	74	43	25
10	0.85	0.85	1.06	1.18	25	39	20	28
11	0.96	1.25	1.30	1.74	35	39	26	28
12	1.31	1.64	2.14	2.64	63	61	39	38
13	1.31	1.74	2.10	2.26	60	30	38	23
14	1.22	1.41	1.59	2.11	50	73	23	33
15	1.12	1.25	1.50	1.58	34	23	25	21
Mean	$9.8 \pm 2.22\%$		$9.5 \pm 1.79\%$		$13.1 \pm 2.97\%$		$6.7 \pm 1.64\%$	

The individual variation for the 15 duplicate assays ranged from 0–33%. The variation was below 15% for 13/15 and above 15% for 3/15 for the original determination of specific activity. The companion assay in the presence of coenzyme Q_3 revealed a range of variation of 3–24%. The variation was less than 15% for 10/15 and above 15% for 5/15. The final and important criterion of deficiency in CoQ_{10} -enzyme activity ranged from 1–20%; the variation was less than 10% for 12/15 and over 15% for 3/15.

Assays on duplicate blood samples from the same patients

Two immediate and sequential blood samples of 10 ml each were drawn from

each of 15 patients into two vacutainers. The blood of each tube was separately processed. In this manner, variables of preparation of the leucocytes could be evaluated in combination with variables of the enzymic steps.

The variation in the specific activity of the CoQ-enzyme showed a mean value of $12.1 \pm 2.59\%$ and the corresponding variation of the assay in the presence of coenzyme Q_8 showed a mean of $16.3 \pm 3.46\%$. The variation in the deficiency of CoQ₁₀-enzyme activity showed a mean of $6.8 \pm 1.38\%$ (Table 2). These mean variations in percent in Table 2 are not significantly different from the corresponding values in Table 1. For example, the mean variations in the specific activities in the presence of coenzyme Q_8 were 16.3 ± 3.46 for the complete assays and $9.5 \pm 1.79\%$ for the enzymic steps alone. However, it does appear that there could be specific variations in the steps for the preparation of the leucocytes.

Table 2. Duplicate assays on two sequential blood samples from the same patient

Patient No.	Specific activity		Specific activity with CoQ ₈		Activation coefficient		Percent deficiency	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd
16	1.40	1.36	2.43	1.93	73	41	42	29
17	1.52	1.31	2.18	1.85	43	41	30	29
18	1.35	1.53	1.90	2.06	41	35	29	26
19	1.19	1.82	1.54	3.45	29	90	23	47
20	1.14	1.16	1.86	1.73	63	49	39	33
21	1.56	1.16	2.25	1.73	44	49	31	33
22	0.77	0.66	1.19	0.73	55	11	35	10
23	1.06	1.34	1.53	1.43	45	7	31	6
24	0.93	0.76	1.47	0.71	58	0	37	0
25	0.77	0.53	0.97	0.64	26	21	21	17
26	1.09	0.59	1.53	0.71	41	20	29	17
27	0.31	0.64	0.51	0.89	65	40	39	28
28	0.55	0.52	0.61	0.82	10	58	10	36
29	0.70	0.99	0.96	1.20	38	22	27	17
30	2.02	1.93	2.36	2.61	17	35	14	26
Mean	$12.1 \pm 2.59\%$		$16.3 \pm 3.46\%$		12.8 ± 2.58		$6.8 \pm 1.38\%$	

Assays of two blood samples from the same patient over weeks of time

It is frequently important to know the variation in the specific activity of this CoQ₁₀-enzyme in the leucocytes of a patient over a period of time. Toward this end, two blood samples were drawn from each of 21 patients over a mean period of 5.6 weeks. The data from these assays reflect primarily upon the metabolic and nutritional variations in the patients during several weeks or months of time. The data are in Table 3.

The mean variation in the specific activity for the 21 patients for periods of

Table 3. Assays on two blood samples from the same patient over weeks of time

Case No.	Time ^a weeks	Specific activity			Specific activity with CoQ ₃			Activation coefficient			Percent deficiency		
		1st	2nd	Var. %	1st	2nd	Var. %	1st	2nd	Var. %	1st	2nd	Var. %
1	3	0.21	1.26	72	0.54	1.41	45	157	12	72	61	11	25
2	3	0.63	1.07	26	1.54	1.77	7	144	65	39	59	39	10
3	3	0.97	0.66	20	1.06	0.49	36	9	0	5	8	0	4
4	3	0.40	1.83	65	1.59	2.20	16	298	20	139	75	17	29
5	3	1.33	0.62	54	1.82	1.20	20	37	93	32	27	48	30
6	4	0.81	0.61	14	1.63	1.06	21	100	74	13	50	42	4
7	4	2.31	1.20	31	3.58	1.53	40	54	27	13	35	22	6
8	5	0.10	0.88	80	0.30	1.34	61	200	52	74	70	34	18
9	6	1.21	0.93	13	1.21	1.13	3	0	22	11	0	18	9
10	6	2.47	0.96	44	2.75	1.37	33	11	43	16	10	30	10
11	6	2.18	2.18	0	3.27	3.77	7	50	73	12	33	42	4
12	6	3.25	1.45	38	7.06	2.75	44	117	90	13	53	47	3
13	6	1.67	1.53	4	3.42	1.53	57	104	0	52	51	0	25
14	7	1.12	1.03	5	1.54	1.89	10	37	80	21	27	45	9
15	7	0.53	1.89	56	0.89	1.89	36	68	0	34	60	0	30
16	8	1.77	0.83	36	1.96	1.01	34	11	22	5	10	18	4
17	8	1.24	1.44	7	0.77	2.31	50	0	60	30	0	38	19
18	9	1.44	1.10	17	1.44	1.00	18	0	0	0	0	0	0
19	10	0.38	0.88	40	0.94	1.47	22	148	67	40	59	40	9
20	12	1.23	0.88	16	1.54	1.41	4	25	61	18	20	38	9
21	4	0.34	1.45	63	0.34	1.91	70	0	36	18	0	26	13
Mean	5.9±0.55	33.4±5.29			30.2±4.30			31.2±6.52			12.9±2.13		

^a between withdrawal of blood samples.

3–12 weeks was $33.4 \pm 5.29\%$, and that variation in the presence of coenzyme Q₈ was $30.2 \pm 4.3\%$. The mean variation in the activity coefficient was $31.2 \pm 6.5\%$, and that variation in the deficiency of CoQ₁₀-enzyme activity was $12.9 \pm 2.13\%$.

On an individual basis over the 3–12 week period, the variation in the specific activity ranged from 0–80%, and the specific activity in the presence of coenzyme Q₈ ranged from 3–70%. The variation in the deficiency of CoQ₁₀-enzyme activity ranged from 0–30%.

Depending upon the purpose which such enzyme data serve, including group analysis, individual patient analysis, guidance of patient selection for possible treatment, *etc.*, it is evident that these enzyme analyses have enough consistency to be useful, particularly when deficiencies of CoQ₁₀-enzyme activity are above 50%.

Assays for correlation of data on leucocytes and muscle

In four other patients, skeletal muscle was obtained by biopsy, and a blood sample was also obtained toward information on the relationship between the specific activities of this CoQ₁₀-enzyme in the leucocytes and in the skeletal muscle

Table 4. Succinate dehydrogenase CoQ reductase of mitochondria from skeletal muscle and leucocytes

Patient	Skeletal muscle				Leucocytes			
	Specific activity	Specific activity with CoQ ₃	Activation coefficient	Percent deficiency	Specific activity	Specific activity with CoQ ₃	Activation coefficient	Percent deficiency
S.D.A.	17.13	21.91	28	22	0.89	1.36	53	35
K.L.E.	25.83	40.29	56	36	1.46	1.70	16	14
T.S.A.	32.11	52.94	65	36	1.37	1.99	45	31
V.H.S.	20.10	39.52	96	49	0.84	1.64	95	48
Mean	23.79 ±3.17	38.67 ±5.34	61 ±14.2	36 ±5.6	1.14 ±0.16	1.67 ±0.12	52 ±16.3	32 ±6.9

of the same individual. The data are in Table 4. There is some agreement between the percent deficiencies of CoQ₁₀-enzyme activities in the leucocytes and in the muscle mitochondrial preparations; the group is small. However, these data constitute promise of useful correlations. The future extension of these data is particularly important, because the obtaining of blood samples from diverse patients is generally far easier than biopsies of skeletal muscle or other tissue.

DISCUSSION

A study has been made of the reproducibility of the entire procedure from the collection of the blood samples through the enzymic steps for determination of the saturation or deficiency of coenzyme Q₁₀ at its site in the succinate dehydrogenase-coenzyme Q₁₀ reductase in mitochondria of leucocytes. Also, the variation of the specific activity in blood samples from given individuals over weeks and months of time, and the relationship between the activities of this CoQ₁₀-enzyme in leucocytes and in other tissues has been explored.

The variation in the determination of the specific activities of the CoQ-enzyme both in the absence and in the presence of coenzyme Q₃ was about 10%. The variation in the determination of the deficiency of CoQ₁₀-enzyme activity was about 7%.

There are, perhaps, greater opportunities for variation, as evidenced by the specific activities in the presence of coenzyme Q₃, for the entire procedure including blood handling, isolation of the leucocyte preparations, and the enzymic steps, than solely in the enzymic steps. This method for obtaining leucocytes is clinically very convenient and practical. However, the leucocyte layer, which is separated from the heparinized blood, does contain other cells including erythrocytes and platelets. These other cells do not contribute significant mitochondrial components, but can contribute additional protein which will be included in the Lowry determination. Such additional protein could be relatively constant in the overall assay, but it is also a basis for another variable in the determination

of specific activity.

According to these data, any variation larger than 32% for a specific activity, 42% in specific activity in the presence of coenzyme Q_8 , and 17% in deficiency of CoQ_{10} -enzyme activity can be recognized as variation with a significance of $p < 0.05$ since these variations represent a mean of ± 2 S.D.

Brin *et al.* (6) found that the percentage change in μg pentose/ml/hr in thiamine deficient rats due to the presence of added thiamine pyrophosphate ranged from 4–25% and the percent depression from the control ranged from 12–43%.

Separate assays of the specific activities on sequential blood samples from the same patient over weeks or months of time showed considerably greater differences than those observed for the data on assay reproducibility. The difference between the specific activities of this enzyme in the leucocytes over a period of time has a significance of $p < 0.005$. The significance of the difference for the corresponding specific activities in the presence of coenzyme Q_8 is $p < 0.001$, and the significance for the differences in activation coefficients and deficiencies in activity corresponds to $p < 0.05$. By such statistics, there were significant differences in specific activities for 10/21 patients, in specific activity with coenzyme Q_8 for 6/21 cases, and percent deficiencies for 7/21 cases. It appears from these data on blood samples from the same patient collected over periods of time that the CoQ_{10} -enzyme activities vary and probably for reasons of metabolism and/or nutrition.

The initial data, toward possible correlation of a deficiency of CoQ_{10} -enzyme activity in the leucocytes and in skeletal muscle, indicate at least some correlation so that the primary exploration for human deficiencies of coenzyme Q_{10} can be based on the analysis of leucocytes with subsequent tissue analysis to be conducted as specifically justified.

Appreciation is expressed to the John A. Hartford Foundation, to the Robert A. Welch Foundation and to Professor O. Wiss of F. Hoffmann-La Roche and Co., Inc., of Switzerland for their respective support. We appreciate the technical assistance of Mrs. Chung-Hee Kim.

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