

Detection of the Level of Energy Metabolism in Patients with Chronic Fatigue Syndrome by Fluorescence Emission from Serum

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Abstract

*In this study we have measured the intensity of serum emission at 450 nm (emission of NAD(P)H) for patients with chronic fatigue syndrome (CFS). Changes in emission of serum in UV-visible range for patients with CFS are compared to other laboratory tests. Results of our analysis demonstrated that: -Bioenergetics measured by serum emission averaged 20% lower in patients than controls. -Serum emission intensity correlated with the concentration of coenzyme Q₁₀ in serum. -Antibodies to infections *Candida albicans* and Epstein-Barr virus (EBV) were elevated in 71%-85% of patients. - There was no correlation between the level of serum emission or energy metabolism and the level of antibodies in serum. -Fatigue was not caused by an under active thyroid; 75% of patients had hormone free T3 in normal range. -Pyrroles concentration in urine was higher than the normal range and correlated inversely with serum emission ($R=0.6$).*

Introduction

Chronic fatigue syndrome (CFS) consists of a combination of nonspecific symptoms—severe fatigue, weakness, malaise, subjective fever, sore throat, a painful lymph nodes, decreased memory and depression, with a remarkable absence of objective physical or laboratory abnormalities.¹⁻⁸ Because of the nonspecific nature of the symptoms and the lack of a diagnostic test, researchers have had difficulty devising a case definition for CFS and, when defini-

tions have been given, they have differed greatly among the various published studies which making direct comparisons of the results obtained in various studies difficult.

For many years researchers have searched for viral causes of CFS. Viruses have been suspected because several viral infections are characterized by a chronic post-infection fatigue and because the onset of CFS often resembles an acute viral illness. However, in other viral infections, the symptoms do not generally persist after several weeks as found in CFS. Because many, but not all, of the patients had Epstein-Barr virus (EBV) antibody profiles that suggested reactivation of latent infection, the syndrome was linked to Epstein-Barr virus. Later it was shown that the serologic associations between the syndrome and cytomegalovirus, herpes, simplex virus types 1 and 2 and measles virus were as strong as or stronger than the association with Epstein-Barr virus. Most of patients with CFS have a high level of EBV antibodies in their blood, but EBV is no longer regarded as the sole or even necessarily a contributing cause for chronic fatigue. Many researchers and doctors consider that the disease involves multiple converging imbalances and deficiencies.

Many studies explored the interrelation of the immune, endocrine and central nervous systems and the possibility that stress and/or reactivation/replication of latent microorganisms could modulate the immune system to induce CFS. It was also proposed that CFS may be caused by immunological dysfunction, for example, inappropriate production of cytokines or altered capacity of certain immunological function. CFS patients showed evidence of

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immune activity demonstrated by increased number of activated T lymphocytes, as well as an elevated level of circulating cytokines.⁹ Immune cell function of CFS patients is poor and immune dysfunction in CFS can be episodic and associated with either physiological and psychological function or activation of latent viruses or other pathogens. Several investigators have reported lower number of natural killer cells or decreased activity of natural killer cells among CFS patients compared with healthy volunteers, but others have found no differences between patients and control group. The results of scientific investigations demonstrate that there are no immune disorders in CFS on the scale traditionally associated with immune disease.

Disturbed mitochondrial function may contribute to or cause the fatigue seen in CFS. Carnitine which is essential for mitochondrial energy production, for example, was reportedly significantly lower in serum.¹⁰ It was demonstrated that CFS patients have significantly reduced serum total carnitine and free carnitine level. However, others¹¹ concluded that serum carnitine does not contribute to CFS.

A number of recent studies showed that oxidative stress might be involved in the pathogenesis of CFS. Oxidative stress can be caused by increased generation of reactive oxygen species. Studies¹² have been reported that erythrocyte morphology was altered in some CFS patients. Certain markers of oxidative stress were elevated and the symptoms of CFS were directly associated with these markers and various measures of oxidative damage. Multiple regression analysis revealed methemoglobin could be used to differentiate CFS patients from control subjects.

A review of research at the 1990 CFIDS Association Research Conference in Charlotte, North Carolina, concluded that the list of what we do not know about CFS is longer than list of the things that are known.

For many years scientists investigating the CFS were convinced that it reflected the immune system's response to an invading infectious agent and that the activity of this microorganism resulted in decreased body energy. Accordingly, we consider that the infectious agent, which is present in patients with CFS, is the result of a weakened immune system. The weakened immune system results in decreased energy metabolism in cells, which is the result of decreased oxygen consumption by cells due to a decrease in oxygen delivery to cells as the result of defects of RBC protection against oxidative damage or aberration of porphyrin metabolism.

As this pathological condition has no diagnostic test and the definition at present is based on signs and symptoms only, we decided to develop a method of measuring the level of energy metabolism for this kind of disorder as a first step to link the level of coenzyme NAD(P)H in serum with clinical tests, which were obtained from patients with CFS. The measurement of fluorescence emission of serum allows an estimate of NAD(P)H and can be used to monitor the alteration in serum emission, which follows changes in the amount of coenzymes in serum, hence changes in the level of energy metabolism.

Experimental Procedures

The patients with CFS were diagnosed according to the Center for Disease Control criteria released in 1994 with a spectrum of symptoms that existed for at least six month: new unexplained, persistent or relapsing chronic fatigue, which was not resolved by bed rest and severe enough to significantly reduce previous daily activity by 50%; headache; muscle pain; pain in multiple joints, and unrefreshing sleep. Other clinical conditions that may produce similar symptoms were excluded by thorough evaluation, based on the appropriate laboratory findings.

Preparation of Samples

Baseline, normal blood samples were obtained from healthy staff volunteers. The experimental samples of serum were obtained from Center patients, which were clinically diagnosed with CFS. Serum was separated from blood by centrifugation at 3500 rpm for 15 minutes. Most serum samples were obtained from individuals who had fasted overnight to exclude the effect of fluorescence emission of drugs and vitamins. Some samples were stored frozen at -20°C for up to one month and measured after this period. To analyze the effect of keeping the serum frozen on fluorescence emission, several samples from healthy volunteers were measured before and after frozen storage and it was shown that the emission of serum did not change after one month.

All serum specimens were diluted by PBS (Phosphate buffered saline) using a ratio of 1:20 to measure the emission in the range of absorption less than 0.1 at the excitation wavelength, which allowed measuring the fluorescence in the region where there is the linear relationship between fluorescence and NAD(P)H concentration. For calibrating the instruments we used several standards: rhodamine B, $[\text{Ru}(\text{bpy})_3]^{2+}$ in EtOH : MeOH (4:1) solution, and a composite of normal serum solutions from several staff volunteers.

Procedure for Measurements

For each specimen and solvent, the fluorescence spectra were run using a SPEX spectrofluorometer (sensitivity 4000:1, double-grating spectrophotometers). The light source was a 125 W xenon lamp. The device had two double-grating monochromators, one for excitation and another for emission. The emitted photons were measured with a Hamamatsu R928 photomultiplier tube. For measurements, the solutions were placed in quartz cells, and the measurements were obtained with a 90-degree angle between the beams of excitation and the emission light path. For the excitation

scans, the range of wavelengths used was 315-340 nm and for emission 330-600 nm. Background curves for the solvent (PBS) were measured each time before measurements of the diluted serum. The fluorescence spectra of solvent were subtracted from the fluorescence spectra of serum to remove background effects.

The blood parameters, level of antibodies and pyrroles in urine were measured by standard techniques used in the Center's certified laboratory for many years.

Results

Spectroscopy: Irradiation of the samples at wavelengths in the range of 300 - 340 nm gave rise to emission in the 350 - 600 nm region, coinciding with the respective absorption and emission of NAD(P)H, along with other contributing molecules. The excitation wavelength was chosen to exclude the effect of protein emission, which has a very intense peak in the range of 280-320 nm. The fluorescence emission spectra for the excitation wavelengths 315 and 340 nm for healthy volunteers' serum are shown on Figure 1 (p.200).

The excitation of samples at wavelengths 340 nm gave better resolution for NAD(P)H, the principle emitting contributor, as will be shown below, and other fluorophore emissions in the visible range since tryptophan, which absorbs at 315 nm, does not have any significant absorption in this range.

Estimation of serum emission for patients with CFS: The fluorescence emission curves for healthy volunteers were measured to establish a baseline for the normal series. Only the serum from fasting volunteers (40 curves) was used for the analysis of emission range variation, as our analysis from non-fasting volunteers showed a significant influence of vitamins (especially vitamin B₆) and drugs uptake on the level of serum emission in UV-visible range.

The average of the curves for 40 healthy volunteers with standard deviations (SD) was calculated for excitation at

315 nm, 325 nm and 340 nm.

To find the differences between serum emission for patients with CFS and healthy subjects, the average normal range was compared with measurements of serum emission from CFS patients. The parameters used for the comparison were: the maximum intensity of emission at 450 nm and the ratio of the intensity at 450 nm to the intensity at 400 nm.

Results are presented in Table 1, (p.201). The values in the columns describe the following parameters for each patient:

-The intensity in counts per second of serum emission at 450 nm at excitation 315 nm (emission of NAD(P)H).

-Percentage difference in the emission intensity of NAD(P)H (maximum intensity at 450 nm) for patients with CFS in comparison with the average emission intensity at 450 nm for healthy volunteers.

-Laboratory values for patients performed at the same serum samples (level

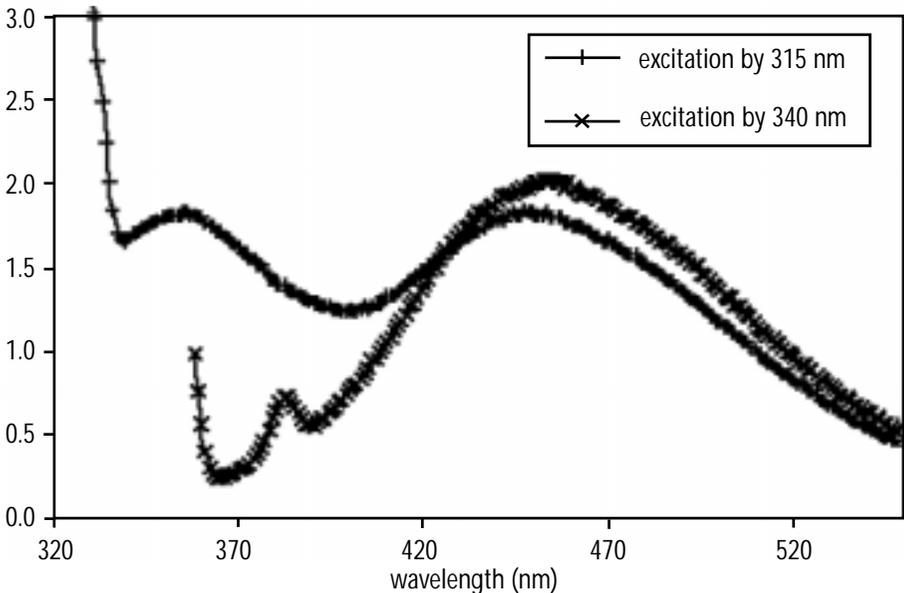
of hormone free T3, CoQ₁₀ level of EBV-early AG (IgG, anti-Candida antibodies in blood (IgG, IgM, IgA), counts of white blood cells and red blood cells, values of hemoglobin, hematocrit, MCV and pyrroles in urine).

A description of the analyses of the serum emission (level of NAD(P)H) for patients with CFS compared to the normal group is presented below. Figure 2 (p.202) contains several curves of serum emission from patients with CFS in comparison with the normal range (mean ± 2 SD).

As shown in Figure 2, (p.202) the emission maximum at 450 nm corresponding to NAD(P)H in serum was usually lower for CFS patients than our normal range. The frequency distributions of the measured intensities at 450 nm (fluorescence of NAD(P)H) for healthy volunteers and for patients with CFS was calculated, normalized on the total number of cases and collectively illustrated in Figure 3 (p.203).

Statistical analysis demonstrated that the

Figure 1. Fluorescence emission of serum of healthy volunteers at two excitation wavelengths.



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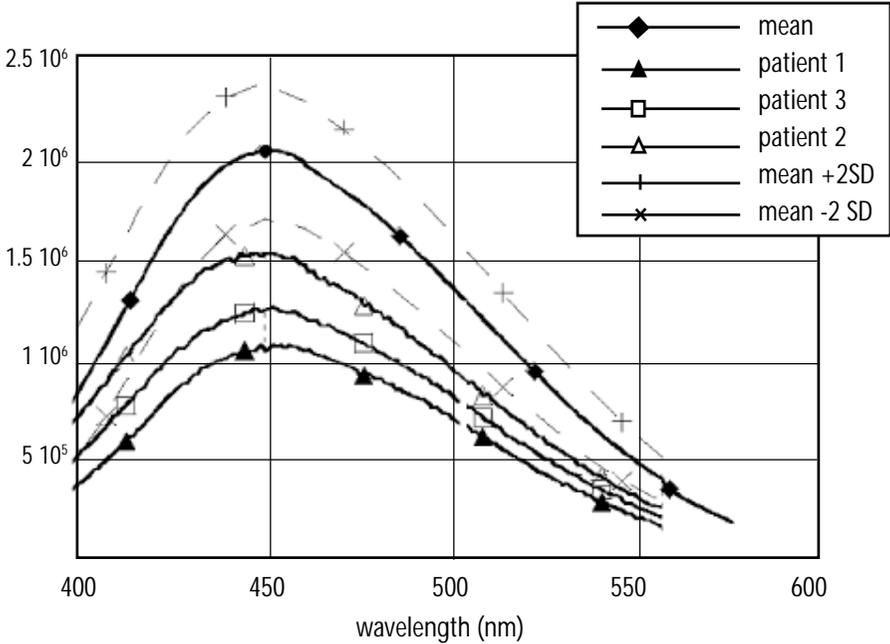
difference between mean values for two groups of data was statistically significant. The level of NAD(P)H and level of metabolic activity were lower for 64 patients with CFS

($P=0.0001$, 2-tailed, mean values of serum emission 1.79×10^6 cps and NAD(P)H 10 nm for 35 healthy controls vs 1.48×10^6 cps and 9.2 nm for patients with CFS).

Table 1. Serum emission and laboratory values for patients with CFS.

Req.#	serum emiss. ($1/10^6$)	% diff	level of NAD(P)H (nm)	Free T3	Q ₁₀	EBV IgG	IgG Candida	IgM antibody	IgA	White Blood Cells	Red Blood Cells	hemo- globin	MCV	hema- tocrit	Pyrroles in urine
50464	0.96	47.30	5.80			39	17	7		5	4.61	13.4	86	39	93
50493	1.54	16.00	9.25	2.66		214	5	5	43						3
53815	1.73	5.46	10.40	5.32	1.8	4	57	19	62	7.4	4.48	13.8			
54153	1.57	14.26	9.44	2.7	1.4		9	14	8	6.5	4.68	14.7	89	41	12
54256	1.56	14.75	9.38	2.33	0.8										11
54315	1.4	23.50	8.42	3.92	1.3	104				6.4	4.85	15.7	90	43	17
54329	1.83	0.00	11.01	3.21	2	55	33	12	36	5.5	3.74	12.4	93	34.9	14
54535	1.65	9.95	9.91	1.95	1.3					7.4	4.78	14.1	84	40.3	8
54654	1.4	23.66	8.40	2.77	1	42	29	18	44	6	4.64	14	86	40	19
54774	1.31	28.14	7.91	3.43	1.3		21	22	33	5	4.05	13.4	93	37	5
54907	1.72	6.01	10.34	2.36	1	127	36	19	7	6.3	4.28	14.4	92	39	3
55017	1.58	13.66	9.50	2.74	1.1	9	4	18	8	5.4	4.43	13	82	36.5	45
55023	1.37	25.14	8.24	2.3											11
55072	1.58	13.66	9.50	2.8	1.2					5.1	4.03	12.8			
55085	1.62	11.48	9.74	3.67	1.2		3	12	10						
55143	1.44	21.31	8.66	2.36	1					6.3	4.28	14.4	92	39	
55158	1.46	20.22	8.78							5.3	4.4	13.9	87	38	
55187	1.97	7.651	1.85							5.3	3.9	12.3			
55413	1.53	16.39	9.20	3.25	1.7		20	19	10	6.1	4.03	12.9			16
55514	1.69	7.65	10.16	3.2	1.9		21	40	13	6	4.64	14.2	87	40	7
55617	1.68	8.20	10.10	3.67			23	22	22	11.8	4.72	14.9	89.8	42.2	16
55657	1.54	15.85	9.26		0.8	46	34	28							
55736	1.05	42.62	6.32							4.8	4.45	14.2			
55823	1.22	33.33	7.34	1.62	0.7		49	18	44						39
55833	1.15	37.16	6.92				21	38	27						
55851	1.6	12.57	9.62	3.64	1.4		60	31	48	6.9	4.71	14.8	89.3	42	8
56156	1.52	16.94	9.14	1.88	1					5.9	4.15	12.5	84	34.9	36
56425	1.66	9.29	9.98	3.16	0.3	23	36	16	8	3.5	4.17	13	90	37	
56487	1.45	20.77	8.72	4.34											16
56514	1.37	25.14	8.24							5.8	4.57	13.5	88	40	
56596	1.05	42.62	6.32	2.95	1.7	37	31	21	47	5.4	4.31	13	89	38.5	8
56599	1.66	9.29	9.98	2.71	1.1	1	53	18	36	6.2	4.62	14	91	41	16
56715	1.09	40.66	6.53	3.26	0.8	12				7.2	5.54	16.9	89	49	108
56833	1.23	32.79	7.40				61	41	43						
56851	1.31	28.42	7.88							9.2	3.46	10.4	92	31	23
56876	1.43	21.86	8.60							6.3	4.41	13.8	91	40.2	19
56897	1.05	42.62	6.32	1.64						7	4.39	13.6	89	39	35
56943	1.76	3.83	10.58	3.12	1.7	25	46	6	32	5.4	5.32	15.5	88	47	14
56968	1.17	36.07	7.04	2.77	0.8	48	55	51	79	2.7	4.06	11.3	84	34	5
56979	1.44	21.31	8.66		1.4		57	26	24	6.9	4.71	14.8	89	42	8
57021	1.39	24.04	8.36				32	20	18						
57298	1.47	19.67	8.84	3.48	1.5	52	14	15	12	3.5	4.38	13.9	90.8	39.6	7

Figure 2. Fluorescence emission curves of human serum for healthy subjects (average \pm 2SD) and for patients with chronic fatigue syndrome.



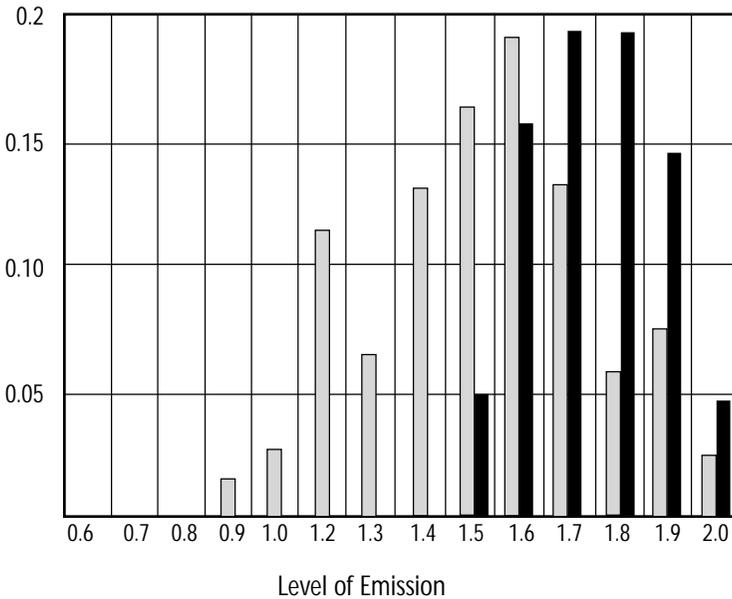
The sensitivity of the method was defined as the percentage of patients with disease whose level of serum emission fell below the reference value (RV), which was chosen as the point of intersection of frequency distribution of emission with the normal distribution curve. The percentage of patients that had emission intensities less than the reference value (equal 1.6×10^6) was for 70% of all patients with CFS. 45% of patients had emission intensities 20% lower than average normal level.

Factor analysis, which was based on the matrix of correlations between all variables, was calculated and presented in Table 1. Emission intensity at 450 nm correlated best with serum CoQ₁₀ concentration (R=0.47) and pyrroles concentration in urine (R=-0.6). The correlation between CoQ₁₀ and serum emission is presented in Figure 4. (p. 204)

An inverse correlation (R=0.6) was found between level of serum emission and level of pyrroles in urine (Figure 5, p.205). The lower level of energy metabolism measured by serum emission, the greater was the detected level of pyrroles in urine. A high level of pyrroles in urine was first described in the urine of patients with various mental illnesses.^{13,14}

We also tested for infectious agents in patients with CSF. In our BioCenter laboratory the test for *Candida albicans*, the microorganism classified as a fungus, was obtained for most of patients with CFS. Another test was the evaluation of EBV-infection. The result of comparison of the level of antibodies in blood due to these infections with the normal range is presented at Figure 6 (p.206). 71%-85% of CSF patients the level of anti-Candida antibodies

Figure 3. Distribution of the level of serum emission at 450 nm (level of NAD(P)(H) patients with CSF and healthy volunteers.



	CSF	Healthy
Mean	1.48	1.79
Variance	0.06	0.017
Observations	64	30
Hypothesized mean dif.	0	
df	90	
t Stat	-7.81	
t Critical one tail	1.66	
P(T<=t) two tail	0.0001	



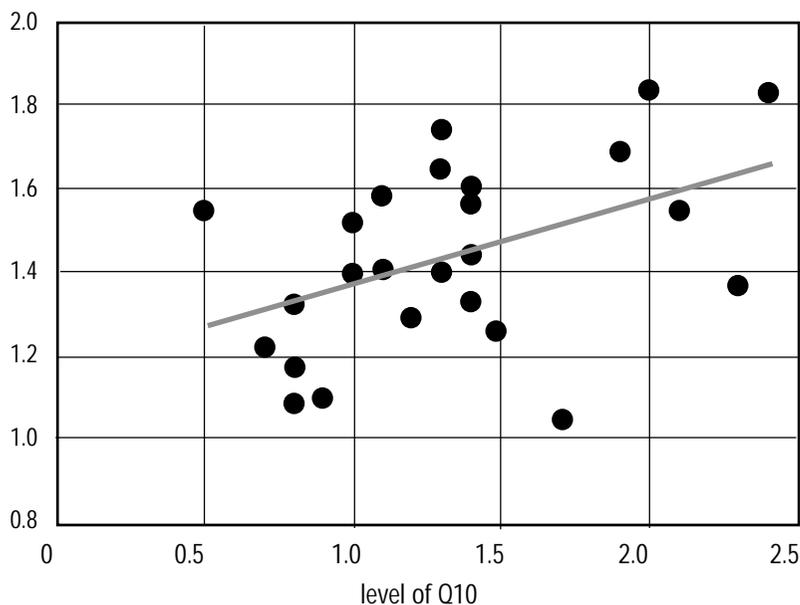
(IgG, IgM, IgA) and level of EBV-early AG (IgG) was higher than normal range.

To test if the condition of CFS is caused by the hormone imbalance, such as hypothyroid, because thyroid hormones play a central role in maintaining the body's energy level and an under active thyroid may cause fatigue, we tested the level of free thyroid hormone T3. For 75% of patients the level of free hormone T3 was in normal range.

We also tested CFS patients for vita-

min deficiencies to determine whether oxidative damage and/or altered protective antioxidant systems might be related to this pathological condition. The level of vitamins for patients with CFS was compared with healthy control. The distribution of vitamin C and vitamin B₆ in serum are shown in Figure 7. (p.207) The figures clearly show difference in the level of antioxidants for these two populations. This result may indicate an increased oxidative stress in patients with CFS.

Figure 4. Correlation between the level of serum emission at 450nm (count per second 10^6) and CoQ₁₀ ($\mu\text{g/ml}$) in serum.

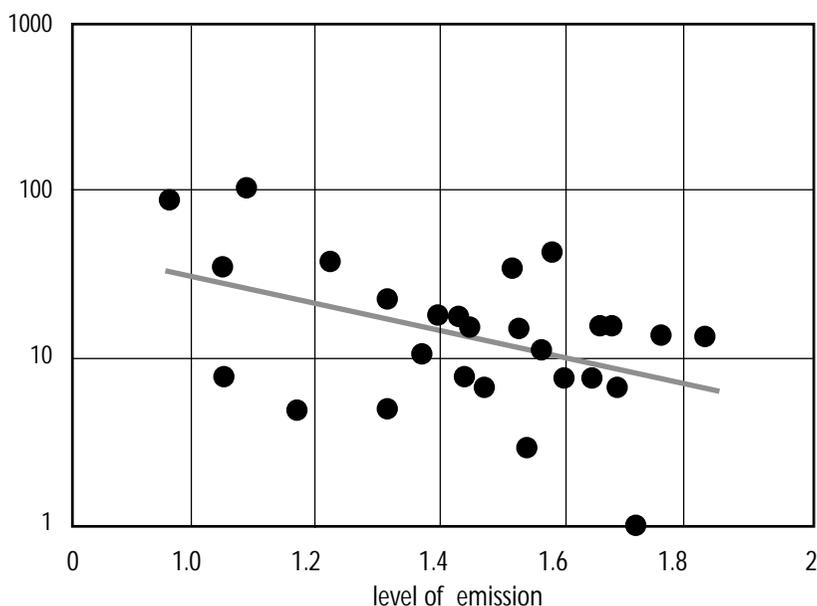


Discussion

Source of Emission: For identifying the different peaks in the serum emission spectrum derived from healthy volunteers and estimating the effect of different fluorescence components (proteins and coenzymes) on the native serum fluorescence, measurements of different fractions of fluorescent serum biomolecules were performed. Others have shown that fluorescence of native serum can be attributed to a variety of molecules such as tryptophan (trp), tyrosine (tyr), phenylalanine (phe), NADH, pyridoxal phosphate, bilirubin, flavin-adenine dinucleotide (FAD) and others. The components used in our analysis were: lyophilized albumin, human (-globulin, 3-hydroxyanthranilic acid, 4-pyridoxic acid, pyridoxal-5-phosphate, and (-nicotinamide adenine dinucleotide (re-

duced form), l-kynurinine, nicotinic acid, nicotinamide (ordered from Sigma). We analyzed the contribution of the different fractions to the emission of serum by using two different methods: by solving linear equations with adjustable coefficients for different components and by estimation of the contribution of the different serum fractions of emission by measuring the emission of serum fraction with real values of concentrations. Comparison of the emission of different fractions proved that the main influence on the native emission at this range of wavelength is from emission of NAD(P)H. Thus, we concluded that changes in emission intensity of serum is due to a decrease of NAD(P)H which correlates and this parameter can be used to monitor the level of energy metabolism in patients with pathological conditions.

Figure 5. Correlation between serum emission at 450nm (cps) and concentration of pyrroles in urine ($\mu\text{g}/\text{dL}$).



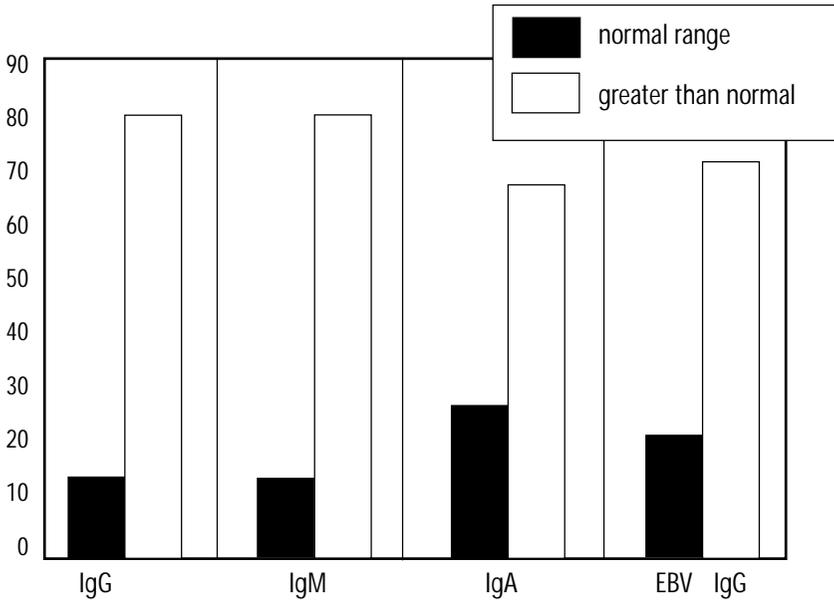
Comparison of Emission to Tests: Serum fluorescence intensity at 450nm (NAD(P)H emission) was lower for patients with CFS than the intensity of emission at the same wavelength for the normal range. Evaluation of the data listed in Table 1 indicates that emission intensity changes in serum do not correlate with counts of RBC or WBC, levels of hemoglobin, hematocrit or MCV, but do show a relationship to CoQ₁₀ in serum and pyrroles in urine.

Coenzyme Q₁₀ is one component of a complex series of reactions that occur within mitochondria. The function of CoQ₁₀ ultimately is linked to the generation of energy within the cells. The reversible oxidation and reduction of CoQ₁₀ is the basis for its function as carrier of electrons between flavoproteins and cytochromes. CoQ₁₀ is essential for ATP production and

for bioenergetics. In comparison with other respiratory carriers in the inner mitochondrial membrane, the content of CoQ₁₀ exceeds the other redox components by about ten fold. The low level of bioenergetics may be due to exhaustion of the store of CoQ₁₀. The correlation between level of CoQ₁₀ and level of serum emission at 450nm (level of NAD(P)H) and lower level of these coenzymes for patients with CFS than for healthy volunteers proves that the bioenergetics is lower for these patients and the proposed method of fluorescence emission measurements are a valid means for energy evaluation.

Emission intensity changes in serum correlate with level of pyrroles in urine. How pyrroles are produced and appear in the urine is still unclear. Our suggestion is that the pyrroles in urine may be results of an

Figure 6. Frequency of abnormal anti-candida antibodies (IgG, IgM, IgA) and EBV early AG (IgG) in serum of patients with CFS.



aberration of porphyrin metabolism (in case of iron deficiency, unstable hemoglobin disease, conditions, which may cause RBC hemolysis, or others) or effect of decreased level of RBC protection against oxidative damage.

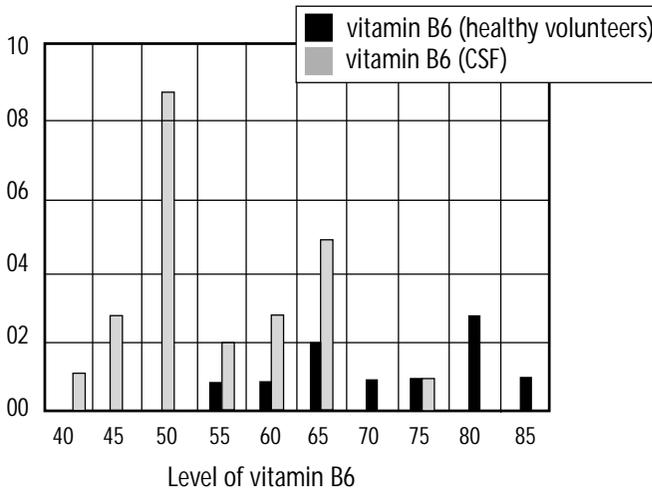
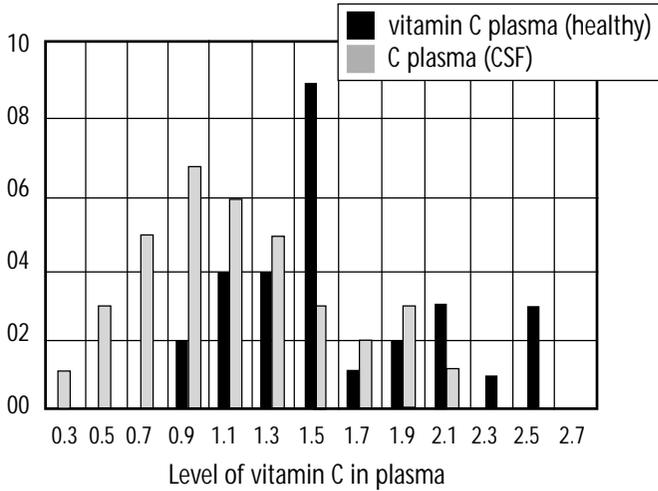
Increased level of methemoglobin (MetHb) in RBC of patients with CFS,¹² which is the product of oxidation of ferrous iron of hemoglobin molecules, also support our results of the decreased level of NAD(P)H in cells, as the formation of MetHb is controlled by NADH-methemoglobin reductase, glutathione and NADH/NADPH. The decreased level of NAD(P)H and increased level of MetHb in RBC for patients with CFS may be associated with oxidative stress and decreased protection of cells against oxidative damage. This hypothesis is also supported by our results about the

level of antioxidants, such as vitamin C and vitamin B₆ in patients with CFS.

The conclusion about lower level of oxygen consumption by patients with CFS was also made in study.¹⁵ Several parameters were measured for these patients: blood volume, peak aerobic power and fatigue level. Peak oxygen consumption was measured during exercise on an upright cycle ergometer and patients displayed a trend for 35% lower peak oxygen consumption ($P < 0.001$).

A non-invasive methodology has been designed in this report to estimate the level of energy metabolism by measuring the fluorescence of reduced nicotinamide adenine dinucleotide (NADH). Our analysis during two years of the level of serum emission for patients with different metabolic disorders, showed characteristic changes in

Figure 7. Vitamin status in patients with CSF.



serum emission for patients from different groups of chronic diseases.

As the results of our analysis we also design the working hypothesis that shows the possible interrelations during the development of this disorder.

Decreasing of the level of energy me-

tabolism and developments of CFS may be result of decreased level of oxygen consumption by cells as the result of decreased level of oxygen delivered to cells by RBC.

Changed level of oxygen transported by erythrocytes and decreased level of oxygen consumption may be caused by aberration

of porphyrin metabolism or by defect of RBC protection by catalase, superoxide dismutase, glutathione or other systems against oxidative damage. This statement was proved by high level of pyrroles in urine, reverse correlation of the level of energy metabolism with level of pyrroles in urine and lower level of antioxidants in serum of patients with CFS. Decreased level of energy metabolism causes the weakening of the immune system. The infectious agents, which are present in the patients with CFS, are the results of weaken immune system.

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