A STUDY OF ACUTE PHASE AND TRANSPORT PROTEIN SYNTHESIS IN UNDERNOURISHED MEN USING SIMULATED INFECTION AND UNIFORMLY ^{15}N-LABELLED *SPIRULINA PLATENSES* (PAPER 1)

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Abstract

This study was conducted to test the hypothesis that acute phase protein synthesis is accelerated and transport protein synthesis is decelerated in adult men in whom the stress of infection is superimposed upon undernutrition. As a pilot study, four chronically undernourished men and two well-nourished controls were studied on two occasions separated by four days; the second session was conducted 24 hours after the administration of typhoid vaccine. Basal urine and blood samples were collected and then subjects were given priming oral doses of ^{15}N-Spirulina (13.5mg/kg body weight) and oral doses (3.5mg/kg body weight) every 30 min for the next six hours. Meals were aliquoted during the dosing period. Blood samples were collected at four, five, and six hours, and urine samples were collected hourly for six hours. ^{15}N enrichment in different fractions of plasma i.e., albumin, non-albumin and amino acids, was measured by combustion GC-IRMS. Total urinary nitrogen was measured by Kjeldahl.

The chronically undernourished subjects had a mean BMI (Wt/Ht^2) of 16.4 ± 1.0 (mean ± SD), and had habitual daily caloric intakes of 1892 ± 299 kcal/day, as assessed by seven day dietary recalls. The controls had a mean BMI of 20.4 ± 0.2 and a habitual daily intake of > 2000 kcal/day. Plasma albumin concentrations were 4.15 ± 0.21 g/dl and 3.625 ± 0.53 g/dl respectively for the controls and undernourished men prior to the vaccination.

^{15}N enrichments increased significantly in all subjects in all fractions of the plasma on both study occasions. In the controls studied before the vaccine, enrichment increased from about 0.38 atom percent at baseline to about 0.58 in the amino acid fraction, and from 0.37 to about 0.40 in the non-albumin fraction. When the studies were repeated 24 hours after typhoid vaccine, the enrichment increased from about 0.39 to about 0.63 in the amino acid fraction in the controls, but increased less in the undernourished, from 0.38 to 0.46. Enrichment increased from 0.40 to 0.44 in the non-albumin fraction in the controls and from 0.39 to 0.42 in the malnourished. In this pilot study, enrichment in the amino acid and non-albumin fractions was higher in the controls than in the malnourished subjects. In the controls, there was a greater enrichment of the

non-albumin fraction (which would include acute phase proteins) after the vaccination. The differences, which were more marked after the vaccination, are due to decreases in the enrichment in the plasma of the undernourished subjects, rather than due to an increased enrichment in the plasma of controls. This latter finding suggests either that malnutrition blunted the acute-phase response to the typhoid vaccine, or that there was greater muscle catabolism in the undernourished, or that there were increased amounts of unlabelled urea in the plasma of the undernourished.

1. SCIENTIFIC BACKGROUND AND SCOPE

Chronic undernutrition is a serious problem in India, where a large proportion of the population have body mass indices (BMI, weight (Kg)/ height^2(m)) which are less than 18.5. These individuals are weight stable, and lead economically productive and otherwise healthy lives. It is possible that this state of chronic undernutrition may influence the susceptibility of such individuals to chronic infections, since it has been shown that the acute phase response is blunted in undernutrition [1].

It is known that undernutrition predisposes individuals to infection, which leads to increased nitrogen loss. The stress of the infection leads to specific changes in metabolism of the body in general and of liver metabolism in particular. There are anabolic responses involved in the mounting of an immune response, consisting of the synthesis and release of globulins and acute phase proteins [2]. This anabolic response is probably maintained by either shifting liver protein synthesis away from other proteins [3], such as transport proteins, or by the catabolism of body proteins such as skeletal muscle. In addition, this catabolism of protein also provides substrates for the synthesis of essential substrates, such as of glucose by gluconeogensis.

Despite the fact that the amino acid sequence of many of the acute phase proteins has been determined [4], the source of the amino acids is not known. There are two possible sources: catabolism of the lean body mass and dietary intake. Since the acute phase proteins are known to have a high content of aromatic amino acids, and since there is an imbalance in the aromatic amino acid content of muscle and acute phase reactants, there would be an excessive breakdown of muscle in order to meet the aromatic amino acid requirements of the acute phase protein synthesis [5]. If the latter is the case, then it may be possible to decrease the catabolism of lean body mass that occurs with infection by increasing intake of protein, aromatic amino acids, or other nutrients. The route of administration of these amino acids is also of interest as parenteral nutrition can be administered when bowel rest is required. In such cases, the entero-insular axis is not activated, and this could lead to different physiological responses by the body. It may also be that selective amino acid supplementation may be more effective in supporting acute phase protein synthesis.

It is necessary to study a model of infection in humans with a view to delineating the components of the stress response as well the nutritional requirements. Stable isotopes offer this possibility. The use of a uniformly labelled protein source, ^{15}N-*Spirulina algae*, minimizes the bias introduced by the use of single tracer amino acids, as well as minimizes the problems of uncertainties with respect to the enrichment of the protein precursor synthetic pool.

2. PREVIOUS WORK FROM THE ST. JOHN'S LABORATORY

Earlier work in this laboratory, done in collaboration with Dr. D. Macallan from St. Georges Hospital, London, measured the kinetics of whole body protein turnover in undernourished male subjects and in newly diagnosed (pre-treatment) tuberculosis patients. ^{13}C-leucine infusions were used in an eight-hour fasted and fed protocol. In that study, it was found that the rates of whole body protein turnover were elevated (by about 8%) in the tuberculosis group. However, this was lower than the turnover measured in similar, healthy but chronically undernourished subjects. It was also lower than the increased rates of turnover in HIV patients (who had turnovers which were 25% higher than controls). Importantly, during the switch from the fasted to the fed state there was a decreased rate of synthesis in the tuberculosis patients compared to controls. This phenomenon needs to be investigated further in the context of the Spirulina study.

It is also necessary to use different probes, such as ^{15}N-glycine, to study whole body protein turnover, which is currently being done in the NRC, Bangalore, by Dr. M.J. Soares. Preliminary results from these studies have shown that the measurement of whole body protein turnover by the ^{15}N-glycine method gives higher values for turnover in the undernourished group. However, this may be related to the dietary intake on the day of the experiment. These two methods, (^{13}C-leucine and ^{15}N-glycine) need to be compared, as the ^{15}N-glycine method is preferable in India, where patients resist blood sampling. Further, the ^{15}N-glycine method gives information on the flux of two different end products: urea and ammonia.

3. OTHER COLLABORATIONS

Apart from this CRP, the Nutrition Research Centre, Bangalore, is also collaborating with the Baylor College of Medicine, Houston, USA in the study of substrate oxidation rates with refeeding in chronic undernutrition. In addition, there is a collaborative effort in establishing the use of the labelled bicarbonate method in the measurement of daily energy expenditure. This collaboration is with Dr. M. Elia, Dunn Clinical Nutrition Centre, Cambridge, U.K.

4. METHODS

Four chronically undernourished, but otherwise healthy young male volunteers, and two well nourished controls were studied. None of the subjects had any significant past history of disease, and all were weight stable. The undernourished subjects had a mean BMI of 16.4 ± 1.0, while the controls had a mean BMI of 20.4 ± 0.2. The basic anthropometry is provided in Table I. The undernourished subjects came from an urban slum, had incomes below the national average, did not have drinking water or sanitation in their homes and were on daily wage labour. Their diets were monotonous, with not much variation from day to day. The caloric intake ranged from 1840 to 1950 kcal/day, and the composition of the diet was as follows: 65 - 75% carbohydrate, 10% protein and the remainder fat. The controls were medical students, with intakes of over 2000 kcal/day.

Ethical approval was obtained from the college ethical board, and informed consent (by thumb print or signature) was obtained from the subjects after the procedures were explained to them in their own language.

The subjects stayed overnight in the laboratory, and were studied the next morning in the fasted state. One hour prior to beginning administration of ^{15}N-Spirulina, subjects were given a meal aliquot. Meal aliquots were then given every 120 min until the fifth hour after the administration of Spirulina. Daily energy requirements were calculated from the 1.56 times the predicted BMR. The BMR was predicted using an equation generated for Indian males in this laboratory. This total caloric requirement was divided into three equal parts, intended for breakfast, lunch and dinner. The first one third of the caloric requirement (for breakfast) was divided into four equal parts, which were given at two hourly intervals, the first part being given one hour prior to the start of the experiment. The other two thirds of the total caloric requirement, were given as lunch and dinner respectively. The aliquoted meal consisted of 75% carbohydrate, 15% fat and 10% protein (soy protein).

The study was started at zero hours (approximately 0700 hours). After taking basal blood samples and emptying the bladder for a basal urine sample, the subjects were given a priming dose of Spirulina (13.5mg/kg Wt). Intermittent maintenance doses (3.5 mg/kg Wt) were subsequently given from 0.5 hours till 5.5 hours at half-hourly intervals (see Fig.1). All urine output was collected at hourly intervals for the first six hours, and at six hourly intervals thereafter until 24 hours. A pooled aliquot was therefore collected for every six hours of the protocol (Fig. 1). Blood samples were collected at four, five and six hours after the administration of the priming dose of Spirulina. The urine samples were acidified and stored at -20°C. The blood samples were anticoagulated with mixed oxalates, centrifuged immediately and the plasma divided into three aliquots. The first and second aliquots were treated with an antiprotease (PMSF, with a final concentration of 100 μ) and stored immediately at -20°C. The first aliquot was shipped to Houston for Apo B100 analysis. The second aliquot was treated as follows: 0.5 ml was taken and the protein precipitated by treating with 6.0% sulphosalicylic acid (SSA) and centrifuged. The supernatant was removed and stored. The precipitate was washed with 6% SSA, and treated with 40% ethanol overnight, after which the precipitate was once again centrifuged and the alcohol removed for analysis. The precipitate was washed with distilled water for subsequent analysis. Therefore three fractions were obtained : the first supernatant (amino acid), the second supernatant of alcohol (albumin fraction) and the third fraction of precipitate (non-albumin fraction). These were analyzed by GC-IRMS (ANCA-MS, Europa Scientific, Crewe, UK). The samples were pipetted in duplicate into tin capsules and dried in a hot air oven. The capsules were then combusted and oxidized in an oxidation furnace (Dumas Combustion), followed by reduction of the oxides of nitrogen to elemental N. This was dried, and fed into the GC-IRMS. Since low enrichments of the fractions were expected, background enrichment ammonium sulphate and albumin were used as standards. The CV of the estimate was 0.07% for ammonium sulphate, and 0.39% for bovine serum albumin.

The urine samples were analyzed for total N by the Kjeldahl technic, and the ammonia and urea enrichments assessed separately.

The fractional protein synthesis rate (FPSR) was calculated from the rate of incorporation of labelled N into individual protein fractions, using the standard precursor-product equation :

$$FPSR = (PE_t - PE_o)/E * 100$$

where (PE_t - PE_o) is the increase in enrichment of protein bound tracer over the period studied, and E is the area under the curve of precursor enrichment.

5. PRELIMINARY RESULTS

The anthropometric and dietary intake data are shown in Table I. Isotopic data are shown in Table II. There was an increase in the ^{15}N enrichment of all fractions of the plasma between the pre-dose baseline and the four, five, and six hour samples on both study days in both groups of subjects. The increase from baseline was highest for the amino-acid fraction, and lowest for the non-albumin fraction in both groups of subjects on both study days. There was a residual enrichment from the pre-vaccination study for all the fractions in the basal post-vaccination study plasma sample. The enrichments of the various fractions after ^{15}N-Spirulina administration seemed to be greater in the controls (Table III). A graphic representation of the time course of the enrichments is given in Figure 2 a and b. The enrichments of the supernatant (amino acid fraction) were fluctuant over the three hours of sampling, though this could have been due to variable absorption along with the aliquot meals. One striking feature was the decrease in the enrichment of the amino acid fraction after vaccination in the undernourished subjects (Fig. 2a). This could be due to an increased amount of unlabelled amino acids reaching the plasma, for example, from muscle protein breakdown, or due to increased amounts of labelled urea in the plasma pool, or to a blunting of the immune response by malnutrition.

6. PLANS FOR FUTURE WORK

It is important to now conduct the ^{15}N-Spirulina study in greater detail and numbers. There are a few recommendations for the next study:

(1) The sample size should be increased from 10 to 12 subjects.

(2) A higher dose of ^{15}N-Spirulina should be used, such that there is a greater enrichment of the various fractions (at least double that seen in this study). This is because the enrichment in the non-albumin fraction was low.

(3) Attempts should be made to measure whole body protein turnover at the same time, using different isotopic labels, eg : ^{13}C-leucine. This is necessary, in order to find out more about the muscle protein turnover during stress.

(4) Some further work needs to be done on regulating the amount of albumin/non-albumin fraction going into the combustion furnace for GC-IRMS. Normally, a good response is obtained with 100 micrograms of sample (albumin). However, with the present extraction method, it is

difficult to regulate this. The Europa system is dependent on constant amounts of sample being combusted.

(5) Other work planned : Since the ^{15}N-glycine method is preferable for measuring protein turnover in India (non-invasive), it is planned to assess this method in relation to ^{13}C-leucine in eight undernourished and eight control subjects. We have already carried out four comparisons, and the samples are being analyzed.

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TABLE I. CHARACTERISTICS OF SUBJECTS

	CONTROLS	UNDERNOURISHED
PARAMETERS		
S.E.S	Class I	Class IV
Height (m)	1.725 ± 0.02	1.646 ± 0.01
Weight (kg)	60.60 ± 0.99	44.50 ± 2.89
B.M.I (Wt/Ht^2)	20.37 ± 0.16	16.44 ± 1.03
Skinfolds	31.60 ± 10.18	18.00 ± 2.51
Fat percent weight	13.05 ± 3.90	6.66 ± 1.65
Fat-free mass, kg	52.72 ± 3.22	41.51 ± 2.21
M.A.C	26.30 ± 0.71	22.45 ± 0.99

Legend

S.E.S. = Socio Economic Scale
B.M.I. = Body Mass Index
M.A.C = Mid Arm Circumference

TABLE IIA. PRE-VACCINATION ENRICHMENTS (atom%)

HOUR:	0000	0400	0500	0600
CONTROLS				
Amino acid-fraction	0.375 ±0.007	0.625 ±0.006	0.659 ±0.025	0.585 ±0.012
Non-albumin (globulins)	0.369 ±0.0006	0.399 ±0.005	0.403 ±0.003	0.396 ±0.007
C.E.D.				
Amino acid-fraction	0.371 ±0.001	0.625 ±0.063	0.659 ±0.023	0.585 ±0.012
Non-albumin-(globulins)	0.372 ±0.002	0.389 ±0.004	0.395 ±0.003	0.401 ±0.002

TABLE IIB. POST-VACCINATION ENRICHMENTS (atom %)

HOUR:	0000	0400	0500	0600
CONTROLS				
Amino acid-fraction	0.386 ±0.003	0.621 ±0.032	0.637 ±0.068	0.631 ±0.051
Non-albumin (globulins)	0.400 ±0.005	0.428 ±0.008	0.429 ±0.007	0.440 ±0.007
C.E.D.				
Amino acid-fraction	0.383 ±0.007	0.473 ±0.016	0.506 ±0.066	0.458 ±0.029
Non-albumin-(globulins)	0.392 ±0.011	0.414 ±0.007	0.415 ±0.007	0.423 ±0.007

TABLE IIIA. PRE-VACCINATION URINARY PARAMETERS

HOURS:	0-6	6-12	12-18	18-24
CONTROLS				
Volume	3973 ± 1241	1485 ± 530	530 ± 456	403 ± 223
Nitrogen	6.63 ± 0	2.52 ± 0.19	1.82 ± 0.87	1.51 ± 0.13
C.E.D				
Volume	2013 ± 688	648 ± 224	398 ± 209	210 ± 34
Nitrogen	2.87 ± 0.98	1.36 ± 0.50	1.24 ± 0.12	1.00 ± 0.23

Volume of urine in millilitres

Urinary nitrogen in grams/litre

TABLE IIIB. POST-VACCINATION URINARY PARAMETERS

HOURS:	0-6	6-12	12-18	18-24
CONTROLS				
Volume	3143 ± 718	468 ± 39	330 ± 127	440 ± 396
Nitrogen	4.49 ± 1.03	0.99 ± 0.39	1.35 ± 0.21	1.55 ± 0.73
C.E.D				
Volume	1121 ± 308	821 ± 623	331 ± 230	195 ± 68
Nitrogen	1.94 ± 0.47	1.39 ± 0.64	1.06 ± 0.61	[illegible] ± 0.45

Volume of urine in millilitres

Urinary nitrogen in grams/litre

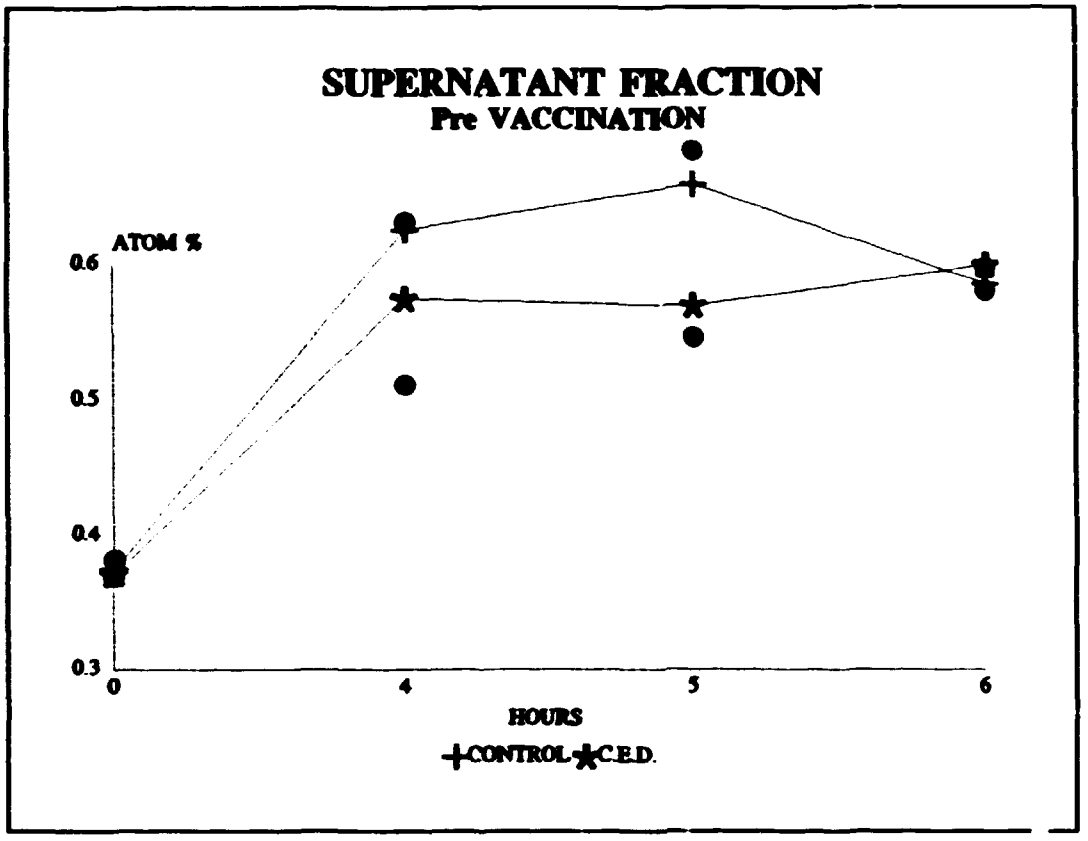

Figure 1 (a)

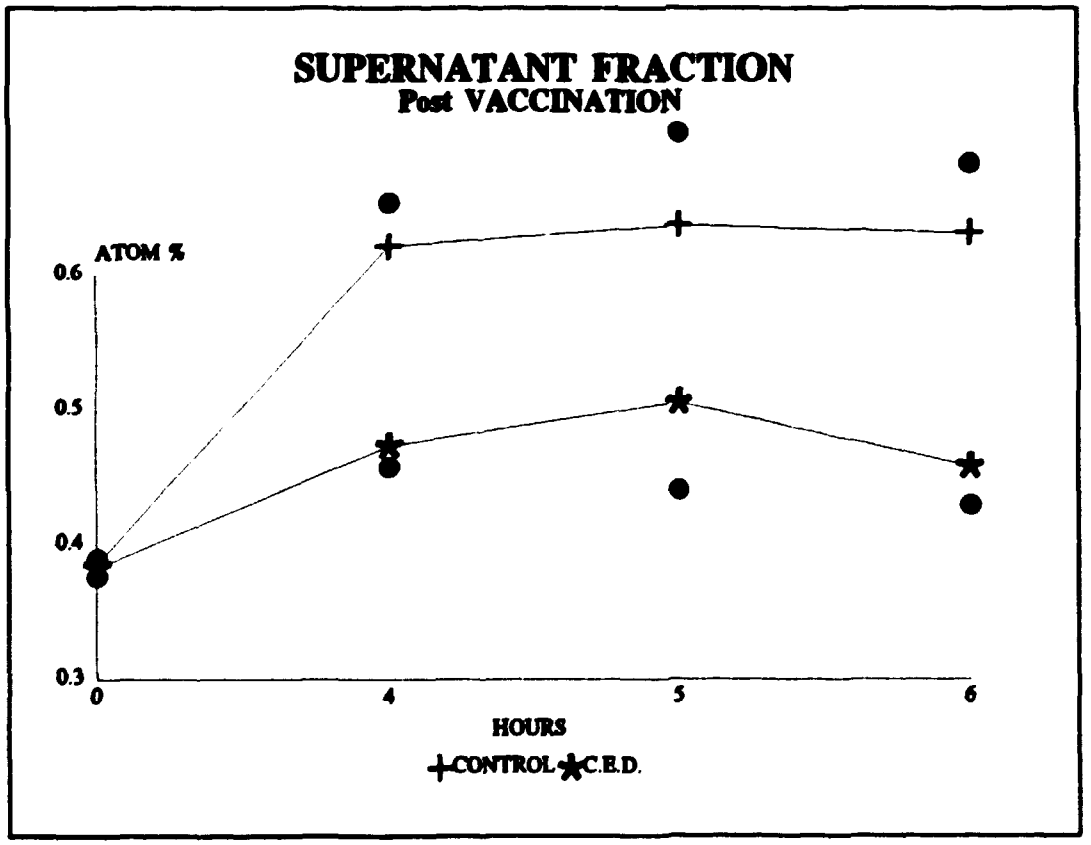

Figure 1 (b)

NON-ALBUMIN FRACTION
Pre VACCINATION

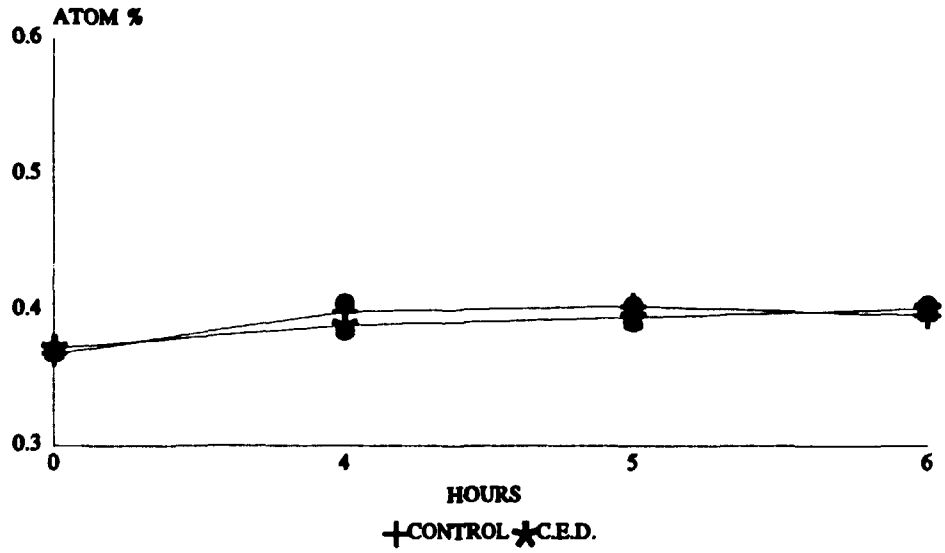

Figure 2 (a)

NON-ALBUMIN FRACTION
Post VACCINATION

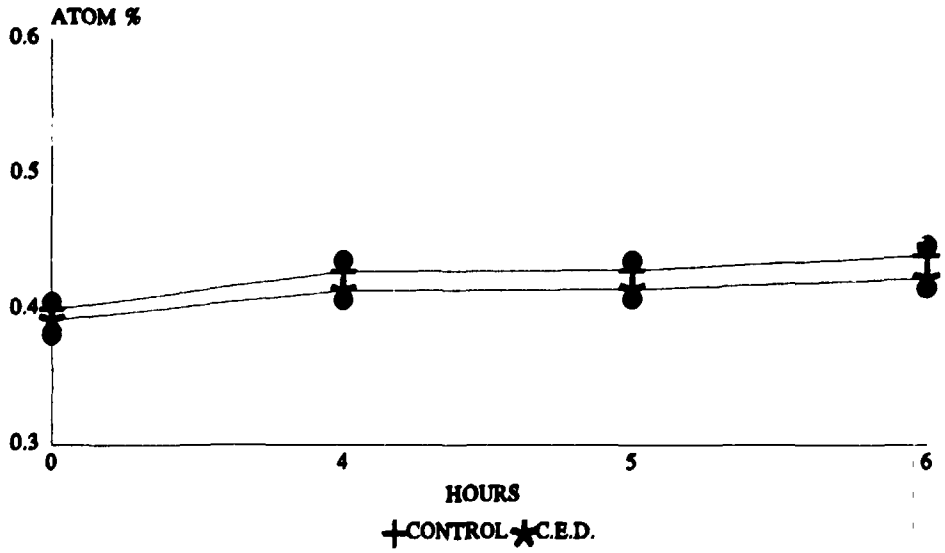

Figure 2 (b)