Gas Chromatography Mass Spectrometry Screening of Urinary Methylmalonic Acid : Early Detection of Vitamin B_{12} (cobalamin) Deficiency to Prevent Permanent Neurologic Disability

Eric J. Norman, Ph. D.

Division of Hematology-Oncology, Department of Internal Medicine, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45267, U. S. A.

Abstract

Screening for vitamin B_{12} (cobalamin) deficiency is described in 68 independently living retirement apartment residents over age 65 through quantitation of urinary methylmalonic acid (MMA) by GC/MS. Five subjects with above normal urinary MMA were evaluated. All five had low serum B_{12} levels without anemia. A second screening study on 85 freeliving church members over age 50 detected three with MMA > 10μg/mg creatinine. Two were evaluated by their physician, determined to have low serum B_{12} levels and were initiated on monthly B_{12} injections. Urinary MMA was also measured in 534 patients suffering from megaloblastic anemia, other anemias, elevated red cell mean corpscular volume (MCV) and/or neurologic disorders. Patients (N = 27) with MMA > 14μg/mg creatinine were confirmed to have a B_{12} deficiency. Data are shown on urinary MMA, serum B_{12}, serum folate, bone marrow, blood counts, and Schilling test results. In addition, a short description of neurologic abnormalities and pertinent history is given. Of 54 verified B_{12} deficient patients detected, 20% had no anemia, 28% exhibited significant mental changes, 52% had a neurologic disability at diagnosis, and many had neurologic manifestations attributable to B_{12} deficiency years prior to

Date of Birth: August 22, 1941
Place of Birth: St. Louis, Missouri U. S. A.
Education :
1961 University of Missouri at Rolla
B. S. (Physics)
1970 Southern Illinois University at Edwardsville
B. A. (Chemistry)
1972 Texas A & M University
M. S. (Biochemistry)
1977 Texas A & M University
Ph. D. (Biochemistry)
Research and Professional Service :
1975- Research and Development Coordinator, Annual Army Reserve Duty, U. S. Army, Natick Development Center, Natick, Massachusetts
1977-1981 Instructor, University above mentioned
1981- Assistant Professor, University above mentioned

diagnosis for B_{12} deficiency. Because of the high prevalance of B_{12} deficiency in the elderly and the possibility of a B_{12} deficient individual suffering a permanent neurologic disability prior to diagnosis, MMA screening of high risk groups is of value. An improved GC/MS method employing deuterated MMA as an internal standard is also described. This procedure, using a single spot urine specimen, offers an efficient means to screen elderly populations for B_{12} deficiency and thereby reduce permanent neurologic disability throught early detection.

Introduction

B_{12} deficiency can be detected reliably through the quantitation of urinary MMA[1] since MMA requires B_{12} for conversion to succinic acid[2]. The prevalence of B_{12} deficiency has been estimated at 2-6 per 1000 in the general population[3] and has been reported to be as high as 4% in a high risk elderly population[4,5].

The onset of symptoms of pernicious anemia is notoriously insidious leading to delays in hospitalization in two studies of 17 months[6] and 14.6 months[7]. More recent work[8-10] including this paper indicate lengthy delays in diagnosis are still encountered. Furthermore, neurologic disease and psychiatric symptoms can occur in the absence of anemia[11-13]. Mental changes of irritability, memory disturbance, mild depression, apathy and fluctuation of mood have been reported in 25-64% of patients with untreated pernicious anemia[14]. Violent maniacal behaviour[12,13] and paranoid psychosis[13] can also be observed in patients with B_{12} deficiency. Peripheral neuropathy and spinal cord involvement can occur in 25% and 75% of untreated patients, respectively[15]. Generally, with therapy, peripheral neuropathy is reversible[15,17] cerebral affection partially remits[14,15,17], and spinal symptoms such as myelopathy seldom improve[15,17]. However, if therapy is initiated within three to six months after onset of neurologic dysfunction, most neurologic deficits will resolve[15].

Early detection of B_{12} deficiency through the means of a simple, noninvasive, sensitive and specific screening procedure would be beneficial for prevention of disability in the elderly[18]. Studies presented in this paper illustrate the feasibility of GC/MS screening of urinary MMA for the early detection of B_{12} deficiency.

Methods

This research was carried out according to the principles of the Declaration of Helsinki, informed consent was obtained and the UCMC Committee on Human Research approved the study.

Morning urine specimens (1-2 ml) were obtained from the 68 elderly retirement apartment residents and the 85 church members participating in the screening studies. Random spot samples were acquired from the 534 patients with megaloblastic or other anemias, elevated red cell MCV and/or neurologic disorders. Urinary MMA[8] and creatinine[19] are stable at room temperature for over 24 hours and for months if frozen, so no special handling of the specimens was required. Urines were assayed for creatinine (Sigma Chemical, St. Louis, MO) using a 20μl aliquot[20]. MMA was quantitated from a volume of urine equivalent to 0.05 mgs creatinine by GC/MS using a Finnigan 3200 mass spectrometer interfaced to a Teknivent 29K data system (St. Louis, MO) programmed from 180°C to 260°C, 15°C/min[8,21]. Quantitation was also performed on an

economically priced Hewlett-Packard 5970A mass selective detector equipped with a 5790 GC having a crosslinked dimethyl silicone capillary column.

The method had recently been improved by using 1μg of the internal standard, methyl-d_3-malonic acid, 99.5 atom % D (MSD Isotopes, Montreal, Canada). Fig. 1 illustrates the mass spectra of dicyclohexyl esters of MMA (a) and MMA-d_3 (b). Fig. 2 depicts MMA quantitation in a patient's urine by monitoring m/z 122 (MMA-d_3) and m/z 119 (MMA) using m/z 101 (MMA) as a confirmation ion. Table I illustrates the within-day and day-to-day precision of the assay. Previous GC/MS methods using deuterated MMA require a time consuming extraction ~2.0 ml of specimen [22~24]. This procedure needs no prior purification using only ~0.05 ml of urine.

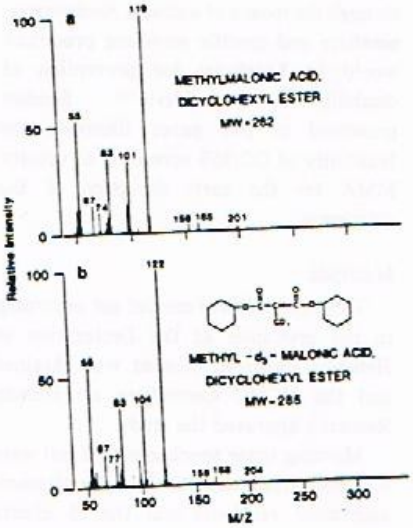

Fig. 1 Electron impact mass spectra of (a) MMA and (b) MMA-d_3, dicyclohexyl esters.

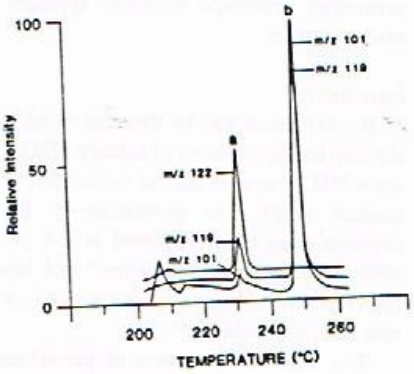

Fig. 2 Selected ion recording from patient's sample. Fig. 2a represents MMA and 2b indicates succinic acid.

Table I Methylmalonic acid quantitation in dilute urine specimens in which MMA had been added. A 50μl aliquot was assayed.

MMA added	MMA (μg/ml) (mean ± S. D., N=5)	
	Within-Day	Day-To-Day
2	2.04±0.13	1.98±0.19
5	5.11±0.15	4.63±0.42
10	10.96±0.96	10.11±0.44

Results

Table II lists the range of concentrations of urinary MMA levels for the 534 patients studied. Levels are expressed as μg MMA/mg creatinine to help compensate for urine dilution. Patients (N=27) with MMA >14μg/mg creatinine were subsequently found to be B_{12} deficient by their physician through diagnostic testing and a return to hematologic normality after treatment with B_{12}. Tables III and IV list laboratory and clinical data recorded in the patient's hospital charts.

Sixty-eight independently living retirement apartment residents over age 65 were then screened through urinary MMA quantitation for early detection of B_{12} deficiency[24]. The resulting MMA concentrations indicated a bell shaped distribution about the 2-3μg/mg creatinine range as noted in Table V. Five individuals outside this distribution with levels >7.0μg MMA/mg creatinine were contacted for additional MMA testing and advised to consult their physician. See Table VI. All five have been evaluated by their physician and are now receiving monthly B_{12} injections. Subject 1, the sister of subject 5, had a MMA of 7.8 even though she had received a B_{12} shot two weeks prior to the test. Two years previously, after feeling tired, she was given a B_{12} shot which improved her health. At that time, she had a normal Schilling test of stage I − 15% and stage II − 18.5% but serum B_{12} levels of 151 and 121. The WHO scientific Group on Nutritional Anaemias (1968) has recommended 200pg/ml as the minimum satisfactory serum B_{12} concentration[17]. Her physician has now elected to give her regular B_{12} therapy. The second stage Schilling test for subject 5 may be low due to transient ileal dysfunction from B_{12} lack which often corrects after one week of vitamin therapy[11].

Table II Range of concentration of urinary MMA in patients

MMA (μg/mg creatinine) Normal <5.0	Number of patients (N=534)
0 − 1.0	115
1 − 2.0	166
2 − 3.0	100
3 − 4.0	55
4 − 5.0	35
5 − 6.0	16
6 − 7.0	7
7 − 8.0	4
8 − 9.0	1
9 − 10.0	0
10 − 11.0	3
11 − 12.0	1
12 − 13.0	4
13 − 14.0	0
14 − 15.0	1
15 − 20.0	1
20 − 25.0	3
25 − 50.0	6
50 − 100.0	4
100 − 500.0	6
500 −1,000.0	4
1000 −2,000.0	2

A similar MMA screening was then conducted at the First Baptist Church of Mt. Healthy, Ohio. This survey screened 85 independently living members of the church over the age of 50 years and their neighbors and/or relatives. Table VII lists the results of the survey. Two subjects have been examined by their physicians. Subject 3, an 81 year old female, with an MMA of 15.0 was forgetful and walking with a walker. She had a serum B_{12} of 73 six months after her MMA test. Subject 5, an 80 year old female with an MMA of 22.1 was undergoing therapy for walking and described as 9° neurotic. She had a serum

Table III Clinical data for B_{12} deficient patients

Patient	Urinary MMA (μg/mg creatinine)	Serum B_{12} (pg/ml)	Schilling Test I	Schilling Test II	Age/Race/Sex	Hematocrit (%)	White cell count (Kmm³)	MCV (μm³)	Platelets (Kmm³)	Bone Marrow	Folate (ng/ml)
1	29	<50	N.T.[a]		72 W F	14.0	2.5	118	39	Meg.[b]	7.6
2	45.4	<83	N.T.		77 W F	38.6	5.5	104.6	301	N.T.	10.9
3	28.8	50	N.T.		69 B M	11.4	5.7	112.5	decreased	Meg.	3.1
4	67	135	N.T.		86 W M	35.8	7.9	102.4	118	N.T.	8.3
5	70.8	>2,400	2.3%		71 B M	28.8	5.8	134	203	Meg.	17.7
6	51.0	60	N.T.		72 B M	30.7	4.1	120.1	181	N.T.	3.1
7	14.0	70	4.3%	17.7%	63 B F	36.8	4.1	84.6	197	N.T.	4.8
8	89.4	<50	3%		69 W F	31.9	4.6	119	190	Meg.	8.4
9	37.9	<100	2.8%		75 W M	43.7	0.2	101	361	Meg.	N.T.
10	128	<100	1.4%	1% 3%(III)	71 W F	25.3	4.6	123	314	N.T.	12.5
11	175	<100	N.T.		55 W M	23.8	4.5	106	142	Meg.	8.1
12	1,700	<100	1%	17.7%	49 W F	21.6	5.4	103	283	N.T.	>16
13	20.6	<100	N.T.		68 W F	32.8	4.6	87	115	not Meg.	15.3
14	400	<100	3%		37 W F	16.4	6.9	109	N.T.	Meg.	6.8
15	17.2	<100	N.T.		82 W F	43.3	8.3	98	N.T.	N.T.	16.0
16	194	1,385	<2%	7%	40 W M	31.2	5.2	118	N.T.	N.T.	8.6
17	162	<100	N.T.		95 W F	32.4	5.2	95	normal	N.T.	7.5
18	29.1	N.T.	N.T.		81 W M	38.0	3.8	108	normal	N.T.	N.T.
19	367	<100	N.T.		56 W M	38.0	6.2	112	309	N.T.	>16
20	694	N.T.	N.T.		70 W M	26	4.7	121	109	N.T.	N.T.
21	917	<50	<1%	20%	36 B M	18	6.2	95.6	198	Meg.	11.2
22	24.8	187,165	21.8%		60 W M	47.0	8.9	85.6	normal	N.T.	3.3
23	33.6	63	8.5%		51 W M	40.1	8.1	94.6	313	N.T.	6.7
24	1,217	<100	1%	14%	59 W M	25.0	3.1	94	115	Meg.	8.3
25	765	61	<3%		61 W F	30.3	3.1	121	222	Meg.	18.3
26	36.6	<100	<2%	18%	24 W M	37.3	4.3	99	233	Meg.	6.5
27	724	<100	2%		91 W M	38.9	7.7	111	normal	Meg.	13.1
Normal	<5.0	180-900 Borderline 100-180	>8			47± 5 (M) 42± 5 (F)	7.8±3.0	87± 8 (M) 90± 9 (F)	150-350		1.8-16

[a] No test
[b] Megaloblastic
[c] 1000μg B_{12} given 10/28/82; MMA (10/29); serum B_{12} (11/2)

Table IV Neurologic abnormalities and pertinent history of B_{12} deficient patients

1. Dementia, loss of memory, walks with walker, history of seizures, diagnosis of PA in 1960's but taken off B_{12} five years ago.
2. Dementia, several months duration, paranoid – "You nurses are going to poison and kill me." Physically aggressive, past psychiatric hospitalization.
3. Dementia, significant deficits of recent memory. CT finds compatible with mild, diffuse cerebral atrophy.
4. Hypersegmented polys, shortness of breath.
5. Dementia, difficulty walking, poor memory and calculation. Became compative when he was to receive B_{12} IM.
6. Dementia, decreasing mental status over two years, paranoid – "Neighbors are spying on me and want me to raise their kids." Loud, hostile, violentprone, unkept, tries to bite and hit, hears voices that frighten her, on B_{12} MCV went to 100.
7. Dementia, paranoid-repeatedly called police about "false burglaries." had numberous traffic tickets and was not paying her bills. MMA went to 2.0 after B_{12}. Forgetful for the past two years, accusatory, confused, some numbness in left ankle.
8. Weakness, depression, weight loss of 20 lbs over 1-1/2 years. Optical degeneration-poor vision. Still has leg muscle weakness after B_{12} therapy.
9. Progressive cerebral palsy – work-up for organic brain syndrome (heavy metal, B_{12}, folate). Difficulty swallowing food and liquids.
10. Visual disturbances, history of peripheral neuropathy, dizziness. After 9 months of B_{12} therapy, still complains of numbness and tingling in lower extremities, staggering when walking, and nervousness.
11. Decreased appetite for 6 weeks, 10-15% weight loss.
12. Marked peripheral neuropathy and cord problems, experienced leg numbness for 10 years, can't button clothes, requires walker and stand-by assistance at all times for safety.
13. No neurological problems, may well have had a B_{12} deficiency without megaloblastic bone marrow.
14. Steady gait with no dizziness or weakness.
15. Weakness right side two days prior to admission. Brain scan abnormal. CT normal.
16. Two weeks tingling in hands and left foot. Weakness, blurry vision, humming in ears, sees flashes of light, divorced four times.
17. Uses walker, confusion and disorientation, family notices changes in mental status.
18. Yearly increase of MCV noted of 87, 90, 97.5 and 102. Had patch of vitiligo for years, mother also had vitiligo.
19. Numbness in hand for 1 year diagnosed as carpal tunnel syndrome. "Hurts to drive a car." Continuing weakness, gait problems and numbness in feet and hands. Brain scan, EEG, and spinal tap. Taste decreased – no flavor. Gait improved after 1 month of B_{12}.
20. Feeling tired, smooth tongue, lemon yellow skin tint, MCV was 98 three years previously.
21. Weakness and orthostatic dizziness for the last 2-3 months, abnormal psychiatric examination.
22. Gradual onset and progression of leg weakness and numbness. Past surgery to decompress lumbar stenosis (2 years ago) and cervical laminectomy (1 year ago) because of pain in arms and cramps in legs. Uses walker, hands quite weak, EMG's very abnormal.
23. Myelopathy for past eight years. Spasticity, jerking of legs so hard he has kicked himself out of bed. Partial gastrectomy (75%) 22 years previously and has dumping syndrome. Degenerative spinal disease believed to be caused by a nutritional B_{12} deficiency. MMA was 4.7 and repeat Schilling test was 18% after B_{12} IM.
24. Decreased sensation in legs and arms for 2 months. MCV was 109 three years previously.
25. Paresthesia in lower extremities, normal gait.
26. Mild mental retardation and poor psychomotor development with a past history of undiagnosed neurologic dysfunction. Myelopathy, spasticity, increasing rigidity of back and lower extremities, unable to relax. One month after B_{12} MMA was 3.2.
27. Unsteady gait with a 2-1/2 year history of falling.

Table V Results of the retirement apartment MMA screening

Range of MMA levels (μg/mg creatinine) Normal < 5.0	Number found (N = 68)	Level of MMA found (μg/mg creatinine)
0 – 1.0	7	
1. – 2.0	15	
2. – 3.0	21	
3. – 4.0	10	
4. – 5.0	7	
5. – 6.0	3	
6. – 7.0	0	
7. – 8.0	1	7.8
8. – 9.0	0	
9. – 10.0	1	9.2
10. – 11.0	1	10.3
11. – 12.0	1	11.7
12. – 31.7	1	31.7

B_{12} of 142 with a normal hematocrit and MCV. Both individuals are now receiving monthly B_{12} injections. Incidently, if three of the seven subjects detected in the two screening studies were prevented 5 years of nursing home care each, the cost savings would amount to $360,000.

Discussion

In a previous study urinary MMA was measured in 1118 patients with megaloblastic or other anemias, elevated MCV's or neurologic disorders and 27 patients with MMA >20μg/ml were confirmed to have a B_{12} deficiency[4,9]. Data analysis from these 27 B_{12} deficient patients and the 27 described in this paper showed at diagnosis 20% had a normal hematocrit, 28% exhibited significant mental changes, and 52% had a neurologic disability which could be attributed to B_{12} deficiency. Some patients suffered neurologic manifestations of B_{12} deficiency for 10, 11, 14 and 25 years prior to diagnosis.

Paranoid psychosis was observed in three slightly anemic patients (2, 6 and 7; Table IV). Early detection and treatment appear to be necessary for correction of B_{12} deficiency mental abnormalities. For example, patients 1 and 2 (Table IV) with several months dementia improved with one month of B_{12} therapy. They were more interactive and less hostile whereas patients 6 and 7 with dementia for over two years showed little improvement after 1 month of B_{12} therapy. Usually mental improvement is complete within a month but some patients continue to improve for several months[14]. The high prevalence of dementia in the elderly, over 5%, may be related to nutritional deficiencies[26]. A recent study on 260 noninstitutionalized elderly has linked decreased memory and abstract thinking ability to low serum B_{12} levels[27]. Reduced B_{12} transport efficiency from the serum to the cerebrospinal fluid may also be a factor contributing to the cause of dementia. The concentrations of several vitamins such as folic acid and ascorbic acid are higher in the cerebrospinal fluid than the plasma[28]; however, cerebrospinal fluid B_{12} levels are only a

Table VI Clinical data on elderly individuals found with elevated urinary MMA in the retirement apartment MMA screening

Subject	Urinary MMA (μg/mg Creatinine) (Normal < 5.0)					Age/Race/Sex			
	4/26	5/4	6/1	7/23	11/15				
1	7.8	5.1	7.5	3.5	N.T.	71	W	F	6/16/83 : Serum B_{12} = 167 (normal 200-900) Hematocrit = 40.0 MCV = 101.2 Combined Schilling test : I − 16.2% (normal 10-42) II − 17.8% Monthly B_{12} initiated 6/16/83
2	9.2	2.3	7.8	8.3	5.4	80	W	F	6/27/84 : Serum B_{12} = 131 Hematocrit = 41.3 MCV = 95.7 monthly B_{12} initiated 7/2/84
3	10.3	2.3	6.0	15.3	2.2	87	W	M	10/4/83 : Serum B_{12} = < 100 (normal 200-1000) Hematocrit = 44.2 MCV = 112 Monthly B_{12} initiated 10/17/83
4	11.7	7.7	8.7	6.3	1.9	71	W	F	5/5/83 : Serum B_{12} 181 (normal 200-900) Hematocrit = 47.5 MCV = 95.2 Monthly B_{12} initiated 6/7/83
5	31.7	7.5	N.T.	12.4*	3.3	74	W	F	5/3/83 : Serum B_{12} = < 100 Hematocrit = 43.9 MCV = 94.6 Combined Schilling test : I − 5.6% (normal 10-42) II − 9.6% Monthly B_{12} initiated 5/3/83

*One day prior to receiving her Cbl injection

small fraction of plasma levels[29]. Patients 22, 23 and 26 (Table III) have severe spinal cord involvement, slight or no anemia, and only moderately elevated MMA levels. They may illustrate the effect of increased MMA concentrations over a prolonged period of time. MMA CoA could substitute for malonyl CoA in fatty acid biosynthesis[30]. The subsequent production and incorporation of these abnormal lipids into the spinal cord myelin has been hypothesized to contribute to the neurologic manifestations of B_{12} deficiency[31~33]. The normal Schilling tests noted for patients 22 and 23 (Table III) and subject 1 (Table VI) could result from impaired assimilation of food B_{12} but not crystalline B_{12}[17,34].

A satisfactory screening test must be specific, sensitive, reporducible, and acceptable to the population being

Table VII Results of the First Baptist Mt. Healthy MMA Screening

Subject	Urinary MMA (μg/mg creatinine) Normal<5.0			Age/Race/Sex	Additional Laboratory Data
	7/24	7/31	8/5		
1	5.6	6.8	—	71 W M	None
2	10.1	—	—	80 W M	None
3*	15.0	4.5	8.0	81 W F	Serum B_{12}=73. (Normal 200-900) (Six months after MMA test)
4	6.9	6.8	—	55 W F	None
5*	22.1	11.6	—	80 W F	Serum B_{12}=142. (Normal 200-900) HCT=39.9. (Normal 33.0-51.9) MCV=88.9. (Normal 60.0-103.0)
6	6.3	—	—	58 W F	None

*Physician has initiated regular B_{12} therapy

screened[35,36]. Furthermore, early diagnosis and treatment should alter the course of the disease[35,36]. In addition, the prevalence of the disease in the screened population must be sufficiently high as to make the test financially justified[35]. Urinary MMA screening in elderly populations by GC/MS appears to meet these criteria. Although a systematic study has not been made to determine the sensitivity of the urinary MMA test, from physician feedback after studying over 1600 patients over a five year period we have not encountered an untreated pernicious anemia patient with normal urinary MMA. A well conducted large scale pilot study should now be undertaken to demonstrate the value of a large-scale population based GC/MS MMA screening program[37].

This study has demonstrated the feasibility of early detection of B_{12} deficiency through urinary MMA screening. B_{12} deficiency without anemia was present in all seven of the screening subjects evaluated and in 20% of the 54 patients studied. MMA quantitation in spot urine specimens provides an accurate, relatively inexpensive, non-invasive approach to prevent permanent neurologic disability in the elderly through the early detection of covert B_{12} deficiency. This work suggests that the establishment of National GC/MS MMA Screening Centers could reduce suffereing in the elderly and their families while providing a large cost savings in preventative nursing home care.

Acknowledgements

I thank Mr. Drue Denton, M. D. for clinical assistance and Mr. M. R. Sorenson and Mrs. J. Clarke for technical assistance.

References

1) Lindenbaum, J. (1983) *Blood* 61, 624
2) Fenton, W. A. & Rosenberg L. E. (1978) *Ann. Rev. Genet.* 12, 223
3) Chanarin, I. (1969) *The megaloblastic anaemias* Oxford, England
4) Williams, E. I., Bennett, F. M. Nixon, J. V., Nicholson, M. R. & Gabert, J. (1972) *Br. Med. J.* II, 445
5) McLennan, W. J., Andrews G. R., Macleod, C. & Caird, F. I. (1973) *A. J. Med. N. S.* 42, 1
6) Panton, P. H. Maitland-Jones, A. G. & Riddoch, G. (1923) *Lancet* i, 274

7) Cox E. V. (1961) *in Europäisches Symposion Hamburg* pp. 590 Enke, Stuttgart
8) Norman, E. J., Martelo, O. J. & Denton, M. D. (1982) *Blood* 59, 1128
9) Norman, E. J., Martelo, O. J. & Denton, M. D. (1983) *Blood* 61, 208
10) Savage, D. & Lindenbaum, J. (1983) *Am. J. Med.* 74, 765
11) Strachan, R. W. & Henderson, J. G. (1965) *Q. J. Med.* 34, 303
12) Smith, A. D. M. (1960) *Br. Med. J.* II, 1840
13) Holmes, J. M. (1956) *Br. Med. J.* II, 1394
14) Zucker, D. K., Livingston, R. L., Nakra, R. & Clayton, P. J. (1981) *Biol. Psych.* 16 (2), 197
15) Roach, E. S. & McLean, W. T. (1982) *Am. Family Physician* 25, 111
16) Chanarin, I. (1979) *The megaloblastic anaemias Blackwell Scientific Publications.* Oxford
17) Roos, D. (1978) *Acta Neurol. Scand.* 69, 59
18) Williams, T. F. (1983) *J. Am. Geriatrics Society* 31, 637
19) Henry, J. R. (1974) *Ed. Clinical Chemistry : Principles and Technics* pp. 549, 2nd Ed., Harper and Row
20) Heinegard, D. & Tiderstrom, G. (1973) *Clin. Chim. Acta* 43, 305
21) Norman, E. J., Berry, H. K. & Denton, M. D. (1979) *Biomed. Mass Spectrom.* 6, 546
22) Montgomery, J. A., Mamer, O. A. & Scriver, C. R. (1983) *J. Clin. Invest.* 72, 1937–1947
23) Zinn, A. B., Hine, D. G., Mahoney, M. J. & Tanaka, K. (1982) *Ped. Res.* 16, 740
24) Trefz, F. K. Schmidt, H., Tauscher, B., Depenc, E., Baumgartner, R. Hammersen, G. & Kochen, W. (1981) *Eur. J. Ped.* 137, 261
25) Norman, E. J. (1983) *Clin. Res.* 31 (3), 686 A
26) Raskind, M. (1983) *JAMA* 249, 2939
27) Goodwin, J. S, Goodwin, J. M. & Gerry, P. J. (1983) *JAMA* 249 (21), 2917
28) Culler, R. W. P. & Spertell, R. B. (1982) *Ann. Neurol.* 11, 1
29) Lazar, G. S. & Carmel, R. (1981) *J. Lab. Clin. Med.* 97, 123
30) Stryer, L. (1975) *Biochemistry* W. H. Freeman and Company
31) Frenkel, E. P. (1973) *J. Clin. Invest.* 52, 1237
32) Kass, L. (1976) *Pernicious Anemia in Smith L. H. J. R., Ed. Internal Medicine,* Vol.7 pp. 63, W. B. Sanders Co., Philadelphia
33) Green, R., McMillan, T., Meyers, G. S., Renshaw, M., Steinberg, N. & Jacobson, D. W. (1983) *Blood* 62, 37 a
34) Lausten, J. & Fallingborg, J. (1983) *Lancet* ii, 518
35) Cochrane, A. L. & Holland, W. W. (1971) *Br. Med. Bull.* 27, 3
36) Logan R. F. A. & Langman, M. J. S. (1983) *Lancet* ii, 667
37) Rowley, P. T. (1984) *Science* 225, 138