Quantitation of Phospholipid Analysis Using P^{32} by a Digital Computer Method in Metabolic Experiments on Niemann-Pick Disease

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Abstract

Methods involving P^{32} uptake into cell cultures, quantitative phospholipid analyses and computerized data analyses have been used to investigate the biochemical defect in Niemann-Pick disease. The results of these analyses indicate a normal synthesis of sphingomyelin, which considering the threefold increase in total sphingomyelin, gives evidence for a decreased ability of cells to catabolize sphingomyelin in Niemann-Pick disease. These methods are applicable to other lipid storage diseases.

Introduction

THE ANALYSIS OF PHOSPHOLIPIDS labeled with P^{32} poses the problem of determining phosphorus in the presence of extraneous lipid and chromatographic material; and the assay of P^{32}, an isotope with a relatively short half-life of 14.3 days. The decay of the P^{32} during the experimental process causes loss of counts and obscures relationships between different experiments.

In this work it was found that a computer program was of value to calculate and tabulate the final data in terms of counts per minute correcting for the decay of the isotope following the time of injection into the experiment, including subtraction of the background from each sample. This computer method is also recommended for the analysis of other relatively short-lived isotopes (6) especially in the biochemical field where time-consuming chemical manipulations of many samples must be made between the initial metabolic experiment and the final chemical analysis of the metabolites.

This paper presents a revision of a method (1) that has given good results for the analysis of total and radioactive phospholipids on small amounts of biological materials. The present study involves phospholipid metabolism in cell cultures derived from normal individuals and from patients with a metabolic lipid abnormality, Niemann-Pick disease.

Experimental Procedures and Data

Tissue Culture Methods

Biopsy specimens were obtained from patients after preparation of the skin site, sternum or iliac crest, with a mixture of 1:1 alcohol:ether. Xylocaine (1%) local anesthesia was used. The biopsy of skin was taken as a small ellipse, followed by the aspiration of bone-marrow with an aspiration needle. The marrow was placed immediately into Leighton tubes containing the growth medium.

The cell cultures were initiated and grown in Eagle's medium with Hanks salts plus 10% calf serum or 10% fetal calf serum with added glutamine, penicillin, streptomycin, and mycostatin. Radioactive phosphate (obtained from the Oakridge National Laboratory, Oakridge, Tenn.) was added to fresh medium which was incubated with cell cultures and controls in duplicate in a 37C water bath. The experiments were ended after incubation for 3 hr by the addition of cold (4C) phosphate buffer.

The cell sheet on a 32-oz prescription bottle was sufficient for one complete phospholipid analysis in duplicate. The cells were first washed four times with 30 cc of cold Dulbecco's phosphate buffer. Then 5 ml of 0.25% trypsin in Dulbecco's phosphate buffer was added and incubated at 36C for 1 hr. The cells were then washed into a 50 cc centrifuge tube using 3 washings of cold buffer (5 cc × 2, 10 cc × 1). The cells were spun down in a 50 cc stainless steel centrifuge tube at 2,300 × *g* for 15 min at 4C and suspended in 5 cc of cold buffer by passing 8 times through a 2 cc disposable plastic syringe with a #20 needle. An aliquot sufficient to fill both sides of a blood cell counting chamber was removed and the cells counted in an area on both sides, counting approximately 500 cells for each cell sheet to be analyzed.

Extraction Procedure

All solvents were redistilled and 1% methanol was added to the chloroform after distillation to prevent decomposition. N_2 was bubbled through the $CHCl_3$:MeOH 2:1 (v/v) before using for extraction or storage of lipids.

A mixture of $CHCl_3$:MeOH 2:1 (7), 20 cc of solvent to 1 g wet weight of tissue, was used for the extraction. Half of the solvent was added to the tissue which was ground finely in a high-speed leak-proof homogenizer (Model OM-1150, Ivan Sorval, Inc. Norwalk, Conn., or Standard model, Lourdes Instrument Corp., Brooklyn, N.Y.) using full speed for 3–5 min with tap-water bath cooling. The resulting mixture was centrifuged in an International Model PR-1 centrifuge at room temperature at 2,400 × *g* followed by suction filtering through Whatman No. 44 paper. Then the extraction was repeated again on the tissue residue including the filter paper.

A check on the efficiency of the extraction procedure was performed by submitting the $CHCl_3$–MeOH extracted residue to continuous Soxhlet extraction with boiling ethanol for 12 hr. In two separate analyses no more phosphorus could be extracted in this manner. The tissue residues were then saponified with 20% KOH, acidified with concentrated HCl to pH 8.0 and extracted twice with ethyl ether. The resulting ether solution was taken to dryness. The brown residue was then extracted and titrated for free fatty acid according to the Dole method (5). Only 1 μeq of titratable acid per gram dry tissue was found in each of two samples.

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The extract was washed as described by Folch et al. (7) using 0.003 N $CaCl_2$. The washed total lipid extract was then evaporated by aspiration in a nitrogen atmosphere at 37C. The lipid was then taken up in a measured volume (usually 2 cc) of $CHCl_3$:MeOH 2:1 and kept in a Teflon-lined screw cap vial at −20C or in a glass-stoppered volumetric flask.

Thin-Layer Chromatography (TLC)

Thin-layer chromatography was performed using model S II (Brinkmann Instruments, Inc., Great Neck, Long Island, New York). Silica Gel G used for TLC was obtained in 10 kg lots for uniformity and reproducibility of analysis. The silica gel was first washed three times with $CHCl_3$:MeOH 1:1 and then filtered in a large Buchner funnel. The silica gel was then oven-dried at 110C for 15 min and cooled for 90 min.

Thin-layer plates, 8 × 8 in., were spread with a 250 μ layer made by mixing 25 g of washed silica gel and 50 cc of distilled water. Plates were air dried for 30 min and then oven dried at 110C for 30 min. This activation was maximal, for higher temperatures and longer times (up to 180C and several hours) did not further activate the plates.

The plates were run in the solvents used by Skidmore and Entenmann (15), employing solvent I (C:M:7 N NH_4OH, 60:35:5) for the first dimension and solvent II (C:M:7 N NH_4OH, 35:60:5) for the second dimension. The plate was sprayed with ninhydrin for location of phosphatidyl serine and phosphytidyl ethanolamine, followed by I_2 vapor identification of all the lipid spots. It was found that if the plates were stained first with iodine and then with ninhydrin, a false positive test for ninhydrin developed. This was followed by Dragendorf reagent (choline test) locating lecithin, sphingomyelin and lysolecithin.

Plates were photographed with a Polaroid MP-3 camera using Type 147 Polaroid film (Polaroid Corporation, Cambridge, Mass.) and a Wratten C-5 No. 47 blue filter.

Phosphorus Determination

The individual spots were scraped with a razor blade on to glassine weighing paper and transferred to 15 cc centrifuge tubes. A modification of the procedure of Bartlett (1) was followed which involved using 0.1 cc of H_2SO_4 for a one hour digestion at 180C; then a few drops of 30% H_2O_2 (Superoxal, Merck) were added and the tubes returned to the heater for 30 min. A repeat addition of H_2O_2 was sometimes needed to complete the oxidation and clear the tube. Then 2.3 ml of 0.22% ammonium molybdate and 0.1 ml of the Fiske-SubbaRow reagent were added. The tubes were mixed thoroughly and heated in boiling water for 7 min. Finally the reaction mixture was centrifuged at 1500 × g for 15 min using a BE-50 International centrifuge to remove the Silica Gel G.

The spots can also be eluted before analyzing the phosphorus. For this method the scraped spots are placed in microcolumns made of capillary pipets plugged with glass wool and eluted first with 5 cc methanol plus 2% water followed with 1 cc of benzene. This elution method gave results which were somewhat more variable than the simple centrifuge method (9).

The experimental procedure is summarized in Fig. 1 and a typical chromatogram is shown in Fig. 2. The ninhydrin-positive spots (#7 and #8) may be lysophosphatidyl serine and phosphatidyl serine.

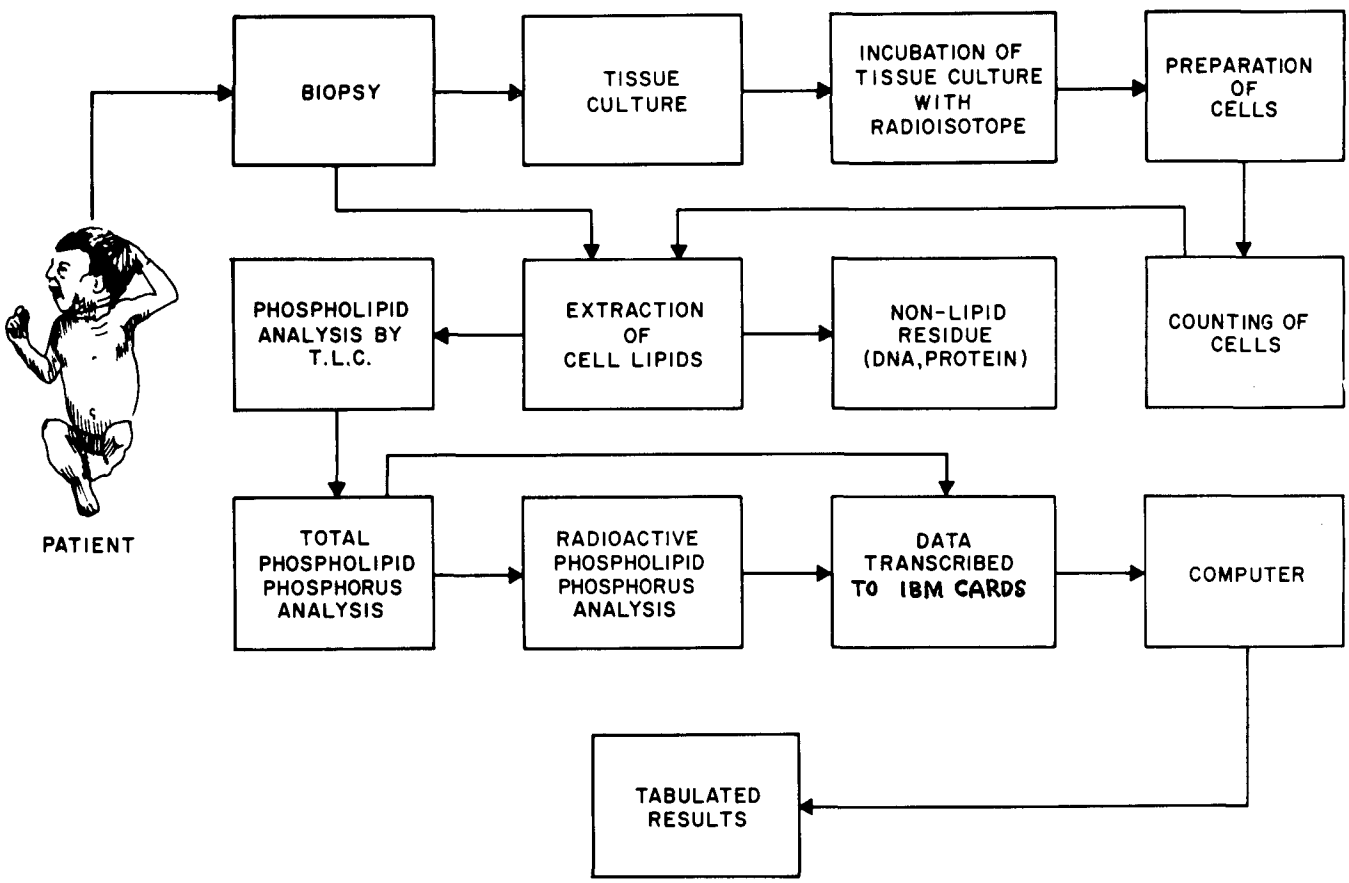

FIG. 1. Flow diagram for metabolic experiments.

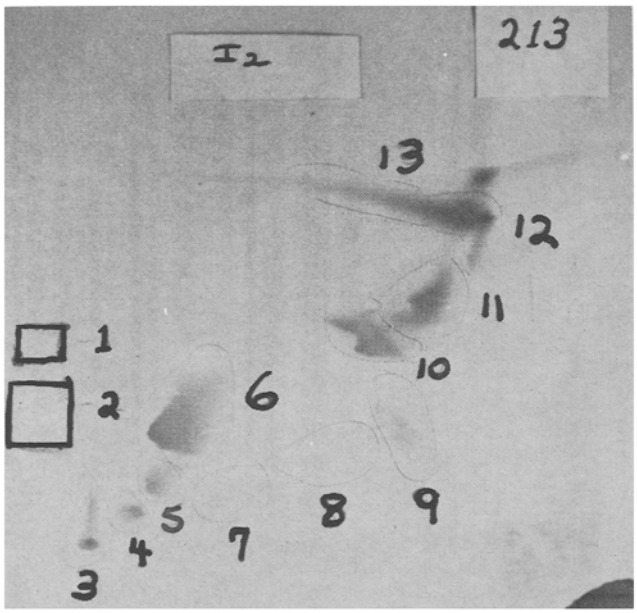

FIG. 2. Normal human liver thin-layer chromatogram. Total load = 11.0 μg of lipid phosphorus.

Legend

1. 1 cm^2 spot used for blank
2. 1 cm^2 spot used for blank
3. Origin
4. Lysophosphatidylcholine
5. Sphingomyelin
6. Phosphatidylcholine
7. Lysophosphatidylserine } analyzed together
8. Phosphatidylserine }
9. Phosphatidylinositol
10. Phosphatidylethanolamine
11. Phosphatidic acid
12. Front
13. Front

There is only one ninhydrin-positive spot seen on the first dimension, which separates into the two spots on the second dimension. These spots have been combined and designated phosphatidylserine.

Purity of Separation

An analysis of the purity of separation was performed. Spots that were separated by TLC were rerun on the same solvent system. Standards of distearyl lecithin and beef spinal cord sphingomyelin were run on the first dimension and then on the second. The R_f values of these compounds agree with the data on the position of sphingomyelin and lecithin. No other spots were identified on these plates following iodine staining, ninhydrin or choline spray reagents.

Thick-Layer Preparative Chromatography

Preparative thick-layer chromatography utilized the same solvents and elution methods as previously outlined for thin-layer analysis. The Brinkmann variable thickness spreader was set at 1.32 mm and the plates were activated longer (2 hr at 110C). It is important to air-dry the plates for at least 1 hr before placing them in the oven to avoid cracking.

The extracts and thick-layer chromatography plates were prepared from human livers as previously described for thin-layer chromatography. The individual phospholipid spots were scraped into small columns and eluted with correspondingly larger volumes (100 cc of MeOH + 2% H_2O followed by 20 cc of benzene). By this method total lipid extracts containing as much as 87.5 μg P or 2.2 mg of phospholipid can be separated into their component phospholipids in discrete spots. These plates gave qualitative separations identical to the separations achieved by the thin-layer method.

Spingomyelin removed from thick-layer plates was rerun on one-dimensional and two-dimensional chromatograms. No choline, ninhydrin and phosphorus were preseent in the sphingmyelin other than in the original area. The test chromatograms were spotted in an atmosphere of CO_2 and run at 4C in an atmosphere of nitrogen to avoid breakdown on the plates.

Radioactive Phospholipid Phosphorus Analysis

A gas flow counter for analysis of P^{32} was used because preliminary experiments with scintillating counter solutions produced varying amounts of quenching, presumably due to the ingredients of the phosphorus reaction.

Plating of the planchet was accomplished by first neutralizing the sulfuric acid in the phosphorus assay with NH_4OH. The plancheting solution consisted of 1 cc of the phosphate analysis, 0.2 cc of concentrated NH_4OH and 0.01 cc of trition 1:5. The solution was mixed in a test tube using a Vortex mixer and two samples of 0.5 cc of the resulting solution were placed on stainless steel planchets, the outer rim of which had been coated with Dow-Corning silicone grease dissolved in ether to prevent creeping.

A standard planchet, made from an accurate aliquot of the original P^{32} solution was included in each day's counting. For the background count, a planchet was made from a blank area of a TLC plate. Counting was performed on a gas-flow counter (Model D-47, Nuclear-Chicago Co.) equipped with an automatic sample changer (Model C-110B, Nuclear-Chicago Co.).

A daily run was begun by noting the exact time of the end of the metabolic experiment, the time the counter began, and the total number of counts to be counted for each sample on the beginning of the print-out tape. After completion of the day's counts, the tape was transferred to the data processor for transcription onto IBM cards.

Computer Program for Radioactive Analysis

A computer program was prepared which incorporated a correction for radioactive decay, and subtracted the daily background from each sample. A Honeywell-800 computer was used. This type of program became necessary when the calculation of background subtraction, counts per minute and tabulation of experimental data became too laborious to be performed manually. The inclusion of a standard P^{32} sample in each run afforded a rapid check of the program since it read essentially the same each day after correction by the computer for radioactive decay.

Program Formula (See addendum for derivation)

The program computes radiation counts for a number of consecutive, equally spaced samples, using the formula:

$$CPM = \frac{CPS \times (TB - Ti) \times 2^{-Ni}}{TB \times Ti} \qquad [1]$$

TABLE I

Phospholipid Pattern of Normal Human Liver (μmoles P/g dry tissue) Repeated Analysis of One Extract by TLC

Date analyzed	Load	OR[a]	LPC	SPH	PC	PS	PI	PE	PA	Front	Total recovery	Recovery percent
10/31/62	85.7	.5	4.7	6.6	33.1	2.8	10.8	20.2	1.4	5.7	85.9	100.2
"	85.7	.9	4.8	8.6	27.4	2.7	11.0	24.8	5.9	6.2	92.3	107.7
11/ 2/62	103.1	2.7	4.4	7.9	64.2	3.8	4.7	17.9	6.3	14.9	126.8	123.0
"	103.1	2.6	4.5	12.7	42.1	12.0	8.4	27.0	4.4	6.6	120.3	116.7
"	103.1	3.1	5.4	8.0	47.4	9.5	8.3	23.6	2.0	6.8	114.1	110.7
11/ 6/62	103.1	1.1	3.5	7.3	38.5	10.6	7.3	19.1	3.9	5.6	96.9	94.0
"	103.1	1.4	4.7	7.2	46.5	13.6	8.5	22.4	5.9	6.4	116.6	113.1
Mean		1.8	4.6	8.3	42.7	7.8	8.4	22.1	4.3	7.5	107.5	109.3
Standard[b] deviation		0.9	0.8	2.1	10.7	1.5	2.1	3.2	2.0	3.1	15.7	9.8

[a] OR—origin, LPC—lysophosphatidylcholine, SPH—sphingomyelin, PC—phosphatidylcholine, PS—phosphatidylserine, PI—phosphatidylinositol, PE—phosphatidylethanolamine, PA—phosphatidic acid.
[b] Standard error of the measurement.

where:

$$-\mathrm{Ni} = \frac{\mathrm{T2} - \mathrm{T1} + (\mathrm{i}-1)\ \Delta\mathrm{T} + \sum_{k=1}^{i} \mathrm{T_k}}{1440 \times \mathrm{t}\ 1/2} \qquad [2]$$

and where (for the i^{th} sample):

CPM = Number of counts per minute
CPS = Number of counts per sample
Ti = Time required for i^{th} sample
TB = Time required for background sample
T1 = Time when experiment ended
T2 = Time when experiment began
ΔT = Time between sample counts
t 1/2 = Half-life of radioactive substance in days
All times are in minutes.

Input to the program is as follows:

Date (day, month, year) and time when experiment ended.
Date and time when counting began.
Number of counts per sample.
Time between samples (in minutes).

The samples are numbered consecutively, and for each sample a sequence number and the time required for the sample is read in. The end of the samples is signaled by two samples with the same sequence number, where the time with the first sample is the time for the background count.

Computer Tabulation of TLC Plates

The use of a computer to record data allows for repeated computations. Additional programs have been devised for the calculation of specific activities and percentage of total recovered radioactivity, tabulated for each TLC plate.

DNA Determinations

Total DNA determinations were performed on the residue following lipid extraction, allowing the phospholipid analysis to be referred to the DNA content of each cell, a quantity that is relatively constant for cell number and type (3). To the residue from the lipid extraction, still contained in the original 50 cc centrifuge tube was added 2 cc of 1 N KOH. The tube was then incubated at 37C with shaking in a water bath to solubilize the residue. Two samples of 0.5 cc each were removed for DNA analyses.

Results and Discussion

Total Phosphorus and Radioactive Phosphorus Analysis

Table I illustrates the reproducibility of the chromatographic separation on a single sample evaluated by phospholipid phosphorus analysis. Presented in Table II are data showing multiple radioactive phosphorus determinations which can be conveniently compared with other experiments although performed with different batches of P^{32} at different times. Niemann-Pick cell cultures are compared with normal cell cultures.

The method here presented is of value in determining phospholipids quantitatively on small amounts of tissue. The entire analysis can be performed on multiple samples in 2 days' time. Thus, many samples can be run on a single specimen and the accuracy of the determination can be increased by the repeated analyses.

A discussion of the problems of incomplete separation of phospholipids by existing methods of analysis by Rouser et al. (14) is of great interest. Although there are difficulties, this method is reproducible and reasonaably accurate; however, more work must be done to define the purity of separa-

TABLE II

Phospholipid Metabolism in Niemann-Pick (NP) Cell Cultures

	(Incubation of P^{32} phosphate for 3 hr)								
	OR	LPC	SPH	PC	PS	PI	PE	PA	Front
Normal sternal marrow (B'n, adult male)									
% incorporation	2.9	N.S.[a]	2.8	52.8	0.3	27.9	18.1	1.2	
P as % of total P			7.3	48.9			20.0		
μg P/100 μg DNA			0.82	2.8		8.1			
N.P. sternal marrow (K.P., female, 2 years old)									
% incorporation	3.7	N.S.	2.7	42.0	0.2	21.1	17.9	0.5	1.4
P as % of total P			13.5	44.7			20.1		
μg P/100 μg DNA			5.9	2.7		7.3			
N.P. Skin (K.P., female, 2 years old)									
% incorporation	0.71	0.16	2.3	48.0	2.0	N.A.[b]	24.4	1.2	0.4
P as % of total P		0.1	16.9	41.9		N.A.	19.0		

[a] Not seen on chromatogram.
[b] N.A. Not analyzed.

tion of the compounds. It should be noted that the original report (15) of this method contained data on hydrolysis and rechromatography of the spots with characterization of the hydrolytic products. By this criteria this method makes good separation.

Further automated procedures that can be used are: 1) The Coulter counter for the automatic counting of tissue culture cells. 2) The Technicon Auto-Analyzer which has been used to replace the tedious phosphorus analysis.

The use of the newer models of gas flow counter with an automatic punch tape and card punch attachment facilitate the transfer of data from the radioactive counter to the computer (13).

Some advantages of using cell cultures to investigate inborn errors of metabolism are: 1) The multiplication of the disease in test-tubes allows extensive experimentation with radioisotopes and toxic chemicals. 2) Many experiments can be performed on cultures from a single patient with a rare disease. 3) The biochemical situation is simplified in cell culture because the physiologic and biochemical spaces are limited to a monolayer of cells bathed on one side by a nutrient medium. The complex in vivo effects of blood flow, intravascular and extravascular space, kidney function, liver function, etc., are avoided. 4) Many generations of cells can be grown in culture, allowing genetic experimentation, not otherwise feasible.

The use of tissue culture to investigate problems of human metabolism has been demonstrated (4,8, 10–12,16). The tissue culture method has the advantage of being useful not only to investigate the cause of inborn cellular metabolic errors but is useful in screening possible drugs and nutritional methods that may be effective in treating these diseases.

Table II shows that despite a high sphingomyelin content in Neimann-Pick cell cultures, the per cent incorporation of P^{32} in the incubation was normal. This gives evidence that the sphingomeylin is synthesized at a normal rate, but is catabolized at a less than normal rate, in agreement with evidence compiled by enzymatic assay by Brady et al. (2).

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Addendum

Formula Derivation

The object of the program is to get a rate count, counts/min, for each of the samples; and to correct all the rates so that they refer to the same moment in time. The moment is the time when the metabolic experiment ends. It is assumed that the nature of the counting process is that each sample is counted until the count reaches a certain fixed number, counts/sec, that is the same for all samples, including the background. The figure recorded for each sample is the number of minutes it takes for the counts from that sample to reach the number CPS. The equipment then feeds another sample into place and starts a new count.

In the following discussion on half-lives the reason for choosing the expression $-Ni$ is clarified. By definition, the count rate of a sample drops in half at the end of one half-life; that is, the rate is multiplied by 2^{-1}. After two half-lives have passsed, the rate is one quarter of its original value; that is, the rate is multiplied by 2^{-2}. In general, the rate is multiplied by 2^{-N} after the passing of N half-lives. The exponent of 2 in the formula, called $-Ni$, represents the negative of the number of half-lives which remain between the time sample i is counted and the end of the experiment. The factor 2^{-Ni} in the formula is this the correction factor which tells what the count rate for the i^{th} sample will be when the experiment ends.

Explanation of the terms of the expression is as follows:

$(i-1)\Delta T$ is the number of minutes taken up by the $i-1$ periods of length ΔT that were taken up by sample changing before the counting for sample i began.

$\sum_{k=1}^{i} T_k$ is the total amount of time, in minutes, used up by counting the i^{th} sample and all the preceding samples.

Thus $(i-1)\Delta T + \sum_{k=1}^{i} T_k$ is the total number of minutes that pass from the beginning of the experiment to the moment when the count for sample i is completed.

Since the experiment ends at time T1 minutes and it began at time T2 minutes. the whole experiment takes $T1 - T2$ minutes. So the part of the experiment that follows the completion of the i^{th} sample is given, in minutes, by:

$$(T1 - T2) - \left[(i-1)\Delta T + \sum_{k=1}^{i} Tk \right]$$

$$= T1 - T2 - (i-1)\Delta T - \sum_{k=1}^{i} Tk$$

$$= - \left[T2 - T1 + (i-1)\Delta T + \sum_{k=1}^{i} Tk \right] \qquad [3]$$

Let us call the time remaining in the experiment after the i^{th} sample is completed Ri. Then by the expression [3], the following can be written:

$$Ri = - \left[T2 - T1 + (i-1)\Delta T + \sum_{k=1}^{i} Tk \right] \qquad [4]$$

To find out how many half-lives there are in a given number of minutes, we divide the number of minutes by the half-life expressed in minutes. If the half-life, t 1/2, is given in days, then to convert to minutes we must multiply by the number of minutes in a day, which is $60\left(\frac{\text{min.}}{\text{hr.}}\right) \times 24\left(\frac{\text{hrs.}}{\text{day}}\right) = 1440\left(\frac{\text{min.}}{\text{day}}\right)$.

So $1440 \times$ t 1/2 is the half-life in minutes, when t 1/2 is specified in days. Thus the number of half-lives represented by the time R_i is $\frac{Ri}{1440 \times t\ 1/2}$. But this number of half-lives (between the end of the i^{th} sample and the end of the experiment) is precisely the definition of Ni, so that:

$$Ni = \frac{Ri}{1440 \times t\ 1/2} \qquad [5]$$

Thus the correction factor, 2^{-Ni}, is equal to:

$$2^{-Ni} = 2^{\frac{-Ri}{1440 \times t\ 1/2}} \quad \text{[by equation 5]}$$

$$= 2^{\left[\frac{T2 - T1 + (i-1)\Delta\ T + \sum_{k=1}^{i} Tk}{1440 \times t\ 1/2}\right]} \quad \text{[by equation 4]} \qquad [6]$$

From here on 2^{-Ni} will be written instead of the whole expression, keeping in mind that the factor 2^{-Ni} is what we multiply the measured count rate for the i^{th} sample by to find out the count rate for that sample at the moment the experiment ends.

Now the basic dimensional equation that gives us what we need is:

$$\left(\frac{\text{counts}}{\text{min}}\right) = \left(\frac{\text{counts}}{\text{sample}}\right) \times \left(\frac{\text{samples}}{\text{min}}\right) = \frac{\text{counts/sample}}{\text{min/sample}}. \qquad [7]$$

If we let:

CPM(i) = counts/min for the i^{th} sample, corrected to refer to the end of the experiment;

CPM′ (i) = counts/min for the i^{th} sample, at the time it was actually measured;

CPM (B) and CPM′ (B) have analogous meanings for the background; and CPM (i + B) and CPM′ (i + B) have analogous meanings for the i^{th} sample plus the background; then we can easily derive the final equation.

We note that the counts recorded by the detector when the i^{th} sample is being run are due to both the sample and the background, which is always contributing. Hence, in accordance with the dimensional equation 7, we can write:

$$CPM'\ (i + B) = \frac{CPS}{Ti}. \qquad [8]$$

The background level is measured at the end of the experiment, so no correction is required; that is:

$$CPM\ (B) = CPM'(B). \qquad [9]$$

Since the background is presumed constant throughout the experiment, we assume that the contribution to the count rate due to the background is CPM (B) for all the samples.

Therefore the count rate due to the i^{th} sample alone is:

$$CPM'\ (i) = CPM'\ (i + B) - CPM\ (B). \qquad [10]$$

The corrected count rate for sample i is then:

$$CPM\ (i) = [CPM'\ (i + B) - CPM\ (B)] \times 2^{-Ni} \qquad [11]$$

Since, in analogy with (8),

$$CPM\ (B) = \frac{CPS}{TB}, \qquad [12]$$

We can substitute [8] and [12] into [11] and obtain:

$$CPM\ (i) = \left(\frac{CPS}{Ti} - \frac{CPS}{TB}\right) \times 2^{-Ni} \qquad [13]$$

$$CPM\ (i) = CPS\left(\frac{1}{Ti} - \frac{1}{TB}\right) \times 2^{-Ni}$$

$$CPM\ (i) = \frac{CPS\ (TB - Ti) \times 2^{-Ni}}{TB \times Ti} \qquad [14]$$

Formula 14 is the same as the formula given in the write-up of the program [see equation 1].