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APPENDIX I

Statistical considerations pertaining to analysis of metabolic models

Donald S. BURDICK

Department of Mathematics, Duke University, Durham, NC 27706, U.S.A.

This Appendix contains the details of the statistical procedures referred to in Crawford & Blum (1983) and in the present study. We first present the formulas that were used and then discuss the rationale behind them.

Notation

Y_{ijk} = the datum from the j th measurement of the k th metabolic product variate for the i th labelled substrate. In the present study,

i varies from 1 ([1- ^{14}C]glucose) to 9 ([2- ^{14}C]acetate), k varies from 1 (alanine) to 8 (lactate), and j varies from 1 to n_{ik} , where n is the total number of measurements of the flow of label from the i th labelled substrate into the k th metabolic product.

$$\bar{Y}_{i \cdot k} = \left(\sum_{j=1}^{n_{ik}} Y_{ijk} \right) / n_{ik}$$

$$S_{ik}^2 = \sum_{j=1}^{n_{ik}} (Y_{ijk} - \bar{Y}_{i \cdot k})^2 / (n_{ik} - 1)$$

$\bar{Y}_{i \cdot k}$ and S_{ik} are the mean and standard deviation, respectively, of the k th metabolic product for the i th labelled substrate. Interval (I or II) and treatment [control (C) or glucagon (G)] may be indicated by superscripts. Thus $\bar{Y}_{8 \cdot 1}^{\text{IG}} = 1.09$ and $S_{8 \cdot 1}^{\text{IG}} = 0.16$ (Table 3). Since calculations are made separately for each of the four treatment cases (IC, IG, IIC, IIG) superscripts are henceforth omitted.

The pooled sample variance for the k th metabolic product, S_k^2 , is given by:

$$S_k^2 = \sum_{i=1}^9 W_{ik} S_{ik}^2 / \sum_{i=1}^9 W_{ik}$$

where $W_{ik} = n_{ik} - 1$ if $n_{ik} \geq 1$ and $W_{ik} = 0$ if $n_{ik} = 0$.

The Between-Substrate Sum of Squares ($BSSS_k$) for the k th metabolic product is given by:

$$BSSS_k = \sum_{i=1}^9 n_{ik} (\bar{Y}_{i \cdot k} - \bar{Y}_{\cdot \cdot k})^2$$

where

$$\bar{Y}_{\cdot \cdot k} = \sum_{i=1}^9 n_{ik} \bar{Y}_{i \cdot k} / \sum_{i=1}^9 n_{ik}$$

The between-substrate variation in the k th metabolic response that is unexplained by the model (UV_k) is defined by:

$$UV_k = \sum_{i=1}^9 n_{ik} (\bar{Y}_{i \cdot k} - C_{ik})^2$$

where C_{ik} is the best fit value from the computer fit to the model of the k th metabolic product for the i th labelled substrate.

The total variation (TV) is defined by:

$$TV = \sum_{k=1}^8 BSSS_k / S_k^2$$

and the total unexplained variation (UV) is:

$$UV = \sum_{k=1}^8 UV_k / S_k^2$$

The total variation that is explained by the best fit to the model (EV) is:

$$EV = TV - UV$$

Statistical analysis

The calculations described above were performed on the transformed data, $Z_{ijk} = \log_{10} Y_{ijk}$. The log transformation was found empirically to improve the between-substrate homogeneity of the within-sample variances S_{ik}^2 . The log transformation can also be supported theoretically because location-invariant statistics in Z_{ijk} are scale invariant in Y_{ijk} . Statistical analysis was performed to address the following four questions.

1. Can the between-substrate variation be attributed to measurement error?

This question can be answered separately for each metabolic product by performing a statistical significance test using as test statistic $F_k = BSSS_k / S_k^2 / DF_k$, where $DF_k + 1$ = the number of labelled substrates for which measurements were made of the k th response. The null distribution for F_k is the F distribution on DF_k and $\sum_{i=1}^9 W_{ik}$ degrees of freedom.

2. Can that portion of the between-substrate variation explained by the model be attributed to measurement error?

Since a fit to the model requires the estimation of flux parameters involving interactions between the responses (i.e. measurements of the flow of label into the various metabolic products measured), it is not feasible to answer this question by performing separate statistical tests for each response. If the measurement error variances, S_k^2 , were not themselves subject to random error, and if the responses were statistically independent, then the quantity EV could be used as a test statistic for addressing this question. Its null distribution would be χ^2 with degrees of freedom equal to one less than the number of independent flux parameters estimated (see below).

3. Can the unexplained variation be attributed to measurement error?

The issues raised in connection with question (2) apply with equal force here. If the unexplained variation were entirely due to measurement error and if independence and known variances could be assumed, the quantity UV would have a χ^2 distribution with number of degrees of freedom equal to the number of substrate-product combinations for which data was obtained minus the number of independent flux parameters estimated.

Since neither independence nor known variances can be safely assumed, only descriptive interpretation can be claimed for the calculations performed (with results stated in the text) in attempting to answer questions (2) and (3).

4. Does the model explain a significant proportion of the between-substrate variation?

The ratio of explained variation to total variation is a commonly used descriptive measure of the explanatory power of a model. The fact that

EV/TV was greater than 0.95 for each treatment case in the present study and in that of Crawford & Blum (1983) indicates that the models used were adequate to explain most of the information contained in the data.

APPENDIX II

To obtain an estimate of the possible range of the flux values, each was varied from its best-fit value, keeping all other independent fluxes constant, until the fit parameter, *E*, increased by 5%. This was considered to represent a significant worsening of the fit, as discussed by Crawford & Blum (1983). Table 1 of Appendix II presents the reaction name(s) and the range for each flux in control and in glucagon-treated hepatocytes. Absent upper-limit flux values indicate that the substrates involved in these reactions are

sufficiently close to isotopic equilibrium that there is no worsening of the fit if they are assumed to be in isotopic equilibrium. While the unidirectional fluxes through bidirectional reactions may be loosely determined or indeterminate, the net flux through each reaction is tightly determined and equals the difference between the unidirectional best-fit values. The symbols for the reactions are shown in the left-hand column, with the corresponding enzyme, reaction, or pathway names listed in the second column.

Table 1. Flux value ranges for a 5% increase in fit parameter for control and glucagon-treated hepatocytes

		Flux (nmol/20min per mg of protein)			
Flux	Enzyme(s) or reaction	Control cells		Glucagon-treated cells	
		Interval I	Interval II	Interval I	Interval II
V_G	Glucokinase	71-94	76-98	52-62	72-85
V'_G	Glucose 6-phosphatase	255-305	135-157	287-317	476-490
V_2	Glucose phosphate isomerase	103-333	49-103	77-247	122-272
V'_2	Glucose phosphate isomerase	320-550	172-225	350-520	345-495
V_{12}	Glycogen synthesis	31-38	51-58	17-18	8-9
V'_{12}	Glycogen degradation	16-66	0-4	10-40	213-227
V_1	Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase	21-30	6-11	31-40	25-37
V_3	6-Phosphofructokinase	77-517	153-533	87-297	113 520
V'_3	Fructose biphosphatase	260-700	250-630	320-530	300-520
V_4	Fructose biphosphate aldolase	> 130	> 270	180-3000	230 3000
V'_4	Fructose biphosphate aldolase	> 313	> 367	413-3233	417 3187
V_5	Triose phosphate isomerase	> 462	125-425	> 448	> 205
V'_5	Triose phosphate isomerase	> 400	80-380	> 450	> 230
V_{15}	Lipid glycerol synthesis	2.7-3.4	3.2-3.4	2.6-3.3	2.3 3.2
V_F	Fructokinase, fructose biphosphate aldolase and triose kinase	248-252	139-150	230-238	162 167
V_6	Lower Embden-Meyerhof pathway	319-404	286-396	269-394	145 435
V'_6	Lower Embden-Meyerhof pathway	175-260	180-290	250-375	175 465
V_R	Ribokinase	23-29	27-32	21-25	20 26
V_7	Ribulose phosphate 3-epimerase	31-37	24-27	36-42	32 40
V'_8	Ribose phosphate isomerase	6-12	14-19	1-5	2 8
V_9	Transketolase	> 17	> 13	> 20	> 18
V'_9	Transketolase	> 0	> 0	> 0	> 0
V_{10}	Transaldolase	35-700	0-280	> 150	80-600
V'_{10}	Transaldolase	52-717	13-293	> 170	98-618
V_{11}	Transketolase	> 19	> 13	> 60	> 28
V'_{11}	Transketolase	> 2	> 0	> 40	> 10

Table 1-(cont.)

Flux	Enzyme(s) or reaction	Flux (nmol/20min per mg of protein)			
		Control cells		Glucagon-treated cells	
		Interval I	Interval II	Interval I	Interval II
V_{PCK}	Phosphoenolpyruvate carboxykinase	95-120	130-172	115-130	86-114
V_{PK}	Pyruvate kinase	239-264	236-278	134-150	56-84
V_{ME}	Malic enzyme	57-99	-52-(-4)	17-35	55-435
V_{PC}	Pyruvate carboxylase	98-140	103-142	56-70	120-500
V_{PDH}	Pyruvate dehydrogenase	61-64	71-75	72-74	79-84
V_{LDH}	Lactate dehydrogenase	208-224	101-123	97-113	9-19
V_{CS}	Citrate synthase	82-85	93-98	88-90	102-108
V_{CIC}	Aconitate hydratase	69-75	84-90	70-78	81-93
V_{ID}	Isocitrate dehydrogenase	69-75	84-90	70-78	81-93
V_{AKS}	Oxoglutarate dehydrogenase and succinyl-CoA synthetase	88-102	80-88	91-100	81-93
V_{SM}	Succinate dehydrogenase and fumarate hydratase	88-102	80-88	90-100	81-97
V_{MDM}	Mitochondrial malate dehydrogenase	> 336	> 149	201-2526	0-195
V'_{MDM}	Mitochondrial malate dehydrogenase	> 400	> 175	175-2500	105-300
V_{MM}	Malate (mitochondria)→malate (cytosol)	> 126	> 112	> 170	> 193
V'_{MM}	Malate (cytosol)→malate (mitochondria)	> 0	> 0	> 100	> 0
V'_{MDC}	Cytosolic malate dehydrogenase	112-137	161-203	117-132	97-125
V_{CL}	ATP-citrate lyase	9-14	6-11	11-19	12-24
V_{AM}	Mitochondrial acetyl-CoA synthetase	19-23	19-24	15-17	21-27
V_{AC}	Cytosolic acetyl-CoA synthetase	17-23	17-25	12-18	6-13
V_{AH}	Cytosolic acetyl-CoA hydrolase	27-33	24-31	25-32	20-33
V_{FA}	Fatty acid synthesis	0.9-1.4	1.9-4.1	0.4-0.5	0.8-1.2
V_{AL}	Alanine input	214-220	126-139	196-204	85-102
V'_{AL}	Alanine output	141-148	42-54	121-129	0-4
V_{GLT}	Glutamate input	60-73	0-5	52-60	0-10
V'_{GLT}	Glutamate output	39-47	2-6	31-37	0.3-3.3
V_{ASP}	Aspartate input	33-54	4-24	78-115	61-123
V'_{ASP}	Aspartate output	0-12	4-23	15-52	26-28
V_{GPT}	Glutamate-pyruvate transaminase	> 1679	318-4918	> 1225	> 4
V'_{GPT}	Glutamate-pyruvate transaminase	> 1750	400-5000	> 1300	> 95
V_{GOT}	Glutamate-oxaloacetate transaminase	25-118	> 39	107-567	53-828
V'_{GOT}	Glutamate-oxaloacetate transaminase	0-150	> 0	90-550	25-800
V_{GDH}	Glutamate dehydrogenase and urea cycle	65-70	38-42	78-82	61-65
V_{FM}	Cytosolic fumarate hydratase	65-70	38-42	78-82	61-65