DEPARTMENT OF THE AIR FORCE
AIR FORCE RESEARCH LABORATORY
WRIGHT-PATTERSON AIR FORCE BASE OHIO 45433

3 April 2002

MEMORANDUM FOR EASTERN RESEARCH GROUP, INC.
100 HARTWELL AVENUE
LEXINGTON, MA 02421
ATTN: KATE SCHALK

FROM: Rebecca A. Clewell
AFRL/HEST
Operational Toxicology Branch
2856 G St, Bldg 79
Wright-Patterson AFB, OH 45433-7400

SUBJECT: Consultative Letter, AFRL-HE-WP-CL-2002-0005, Response to issues raised by the EPA External Peer Review regarding the physiogically-based pharmacokinetic models for perchlorate and iodide.

1. On March 5 and 6, 2002, the U.S. Environmental Protection Agency convened an external peer panel to critically review the proposed risk characterization for perchlorate (ClO_4^-) (USEPA, 2002). The purpose of this response is to clarify several issues raised at this meeting regarding the physiologically based pharmacokinetic (PBPK) models for perchlorate and iodide.

2. The peer review panel expressed concerns over the assumption that ClO_4^- was transferred into the thyroid cell via the sodium iodide symporter (NIS), the model description of passive diffusion of iodide in the thyroid follicle, and the incorporation of iodide uptake in the placenta and mammary gland. The proposed mode of action that drives the structure of the PBPK models is based on the assertion that ClO_4^- competes with inorganic iodide for NIS binding sites at the basolateral membrane of the thyroid. This binding of ClO_4^- to the NIS prevents iodide transport by the symporter and allows the perchlorate anion to enter thyroid cells. This report presents support for this mode of action, particularly the accumulation of ClO_4^- against a concentration gradient, the transfer of ClO_4^- by NIS and the translocation of ClO_4^- into the thyroid cell, as well as other issues raised by the peer review panel.

3. Perchlorate has been measured consistently in rat thyroids at concentrations as much as 30 times greater than the serum.

a. The possibility of analytical interference by other metabolites is ruled out by the following:

i. Double-labeled $^{36}Cl^{18}O_4^-$ studies show no appreciable metabolism in the rat; greater than 99.9% of ClO_4^- dose was unchanged in the urine. Less than 1/1000th of the dose constituted total possible metabolites, including ClO_3^- and Cl^- (Anbar *et al*., 1959). See Attachment 1 for further explanation.

ii. The ion chromatography method used to measure cold ClO_4^- in the thyroid, serum, urine and other tissues of the rat easily distinguishes between ClO_4^- and other possible reduction products, such as chlorate (ClO_3^-) and chloride (Cl^-) (see Attachment 2).

iii. Measurements of cold ClO_4^- have shown over 90 and 88% of the dose excreted in urine of rats and humans, respectively, within 24 hours of dosing (Yu *et al*., 2000a; Merrill *et al*., 2001a).

4. The transfer of ClO_4^- into the thyroid is most likely accomplished by NIS.

a. Perchlorate as a true competitive inhibitor is supported by the mutual inhibition of iodide and perchlorate. Iodide and other iodide inhibitors have been shown to inhibit $^{36}ClO_4^-$ thyroid uptake (Anbar *et al*., 1959; Lewitus *et al*., 1962; Wolff, 1998).

b. ClO_4^- is consistently concentrated in tissues with active NIS, including milk, GI contents and skin, in both drinking water and *iv* studies (Yu *et al*., 2000a, b) (Attachment 3).

c. Thyroid ClO_4^- uptake is subject to up-regulation of NIS, showing increased ClO_4^- levels which are directly proportional to the dose-dependent and time-dependent changes seen in iodide uptake (Attachment 4). Short-term studies have also shown increased ClO_4^- uptake in thyroids of rats given TSH (Chow *et al*., 1969; Lewitus *et al*., 1962).

5. Most evidence suggests ClO_4^- is transported into the thyroid cells. It is unlikely that the measured concentrations can be attributed to NIS bound ClO_4^- in the follicular membrane.

a. Chow and Woodbury (1970) found that $^{36}ClO_4^-$ was actively transported into the stroma and follicle quickly after dosing. They further concluded a second transport mechanism, with a longer half-life, was responsible for actively transporting ClO_4^- into the lumen. This behavior was compared to that of chloride; Cl^- was not concentrated in the thyroid cell and lumen against a concentration gradient as was ClO_4^- (Attachment 5).

b. Hildebrandt and Halmi (1981) report that ClO_4^- affects the utilization of iodine within the thyroid cell in addition to inhibiting NIS transport. The authors administered radioiodide to rats 72 hours prior to ClO_4^- dosing and measured the effect on internal iodide measurements. In the presence of propylthiouracil (PTU, which blocks iodide organification) and thyroid stimulating hormone (TSH, which increases uptake of inorganic iodide) significant discharge of the internal iodide was observed as a result of ClO_4^- exposure. This behavior suggests that

ClO_4^- not only enters the thyroid cell, but also inhibits further steps in the organification process.

c. The kinetic behavior of ClO_4^- in the rat from thyroid time course data taken after *iv* dosing suggests the movement of the anion into a deep compartment. In order to simulate this behavior, the ClO_4^- was described as moving into the thyroid lumen. Unlike iodide, it is not bound to thyroid hormones and therefore readily diffuses back into the cell (Attachment 6).

d. Studies by Riedel *et al*. (2001a, b) do not definitively refute the transfer of ClO_4^- into the thyroid by NIS. As the authors themselves discuss, these data are at least consistent with ClO_4^- transport by NIS at a 1:1 stochiometric ratio with sodium (Na^+), as opposed to a 2:1 Na^+:I^- stochiometry (see also Nilsson, 1999; Dohan *et al*., 2000) (Attachment 7).

e. If ClO_4^- were simply bound to NIS and not translocated into the thyroid cell, the thyroid ClO_4^- concentration should equal the number of inhibited NIS. However, measured data show a non-linear dose-response curve for thyroid ClO_4^- concentration vs. inhibition and 100% inhibition grossly under-estimates the maximum thyroid ClO_4^- concentration, indicating that ClO_4^- accumulates in the thyroid cells (Attachment 8).

6. Passive diffusion of iodide in the thyroid follicle provides a means for the radioiodide to leave the thyroid in the absence of a model that accounts for hormone production and secretion. The primary concern raised by the external reviewers was that passive diffusion might outweigh the effect of the active transport in iodide transfer. However, use of a passive transfer mechanism, in addition to the saturable mechanism, is supported by the following:

a. Radioiodide is seen in tissues lacking NIS, such as the fat, muscle and spleen, after *iv* administration, suggesting that the anion is transferred into tissues to some extent via passive diffusion (Yu *et al*., 2000a).

b. Chow and Woodbury (1970) observed that iodide was transferred into the thyroid by passive diffusion when the active transport was blocked.

c. In the model, passive diffusion is not the determining factor for thyroid iodide concentration until the NIS is saturated. Due to the high Km value (4.0×10^6 ng/L) obtained from Gluzman and Niepomniszcze (1983) for NIS transport, the models do not predict saturation of NIS within the range of the available data, which span five orders of magnitude. Thus, relative to the active transport by NIS, the amount of iodide transferred via passive diffusion is negligible.

7. Iodide uptake in placenta was not described with NIS transfer in early versions of the models. Although the developmental models included active transport into the mammary gland via NIS, they did not include a description of active transport in the placenta based on the available time course data. Since submittal of the models to the EPA in June 2001, newly obtained data and further work in model development have led to important changes in the gestation model structure that help evaluate fetal inhibition and transfer kinetics of ClO_4^- and iodide. These

changes have minimal effect on predicted serum dosimetrics; however, fetal inhibition is greatly affected (Attachment 9). The changes in the model include the following.

a. Active uptake of iodide and ClO_4^- in the placenta was added.

b. Inhibition is included in tissues containing NIS other than the thyroid, such as the placenta as well as maternal and fetal skin and GI contents.

8. The strength of these models as predictive tools and the strongest support for the chosen model structures lies in the ability of the models to predict ClO_4^- kinetics in a diverse set of exposure scenarios using, for the most part, identical parameters. The parameters shown in Attachment 10 were obtained from the most recent versions of the models.

9. When taken as a whole, the published literature (Wolff, 1998; Chow and Woodbury, 1970; Hildebrandt and Halmi, 1981; *etc.*) and recent pharmacokinetic experiments (Yu *et al.*, 2000a, b) provide a more complete picture of the biological response to perchlorate exposure. Although the mechanism of action for this anion is still unknown, the weight of evidence suggests that inhibition of thyroid iodide uptake by ClO_4^- is truly competitive and thus is a reasonable foundation for the ClO_4^- induced iodide inhibition PBPK models.

10. References from this letter and the attachments can be found in Attachment 11.

11. The coauthors for this consultative letters are Elaine A. Merrill, Dr. Peter J. Robinson, Teresa R. Sterner, Dr. Jeffery M. Gearhart, Dr. David R. Mattie and Dr. Kyung O. Yu.

12. For further information, please contact me by phone: (937) 255-5150 ext. 3141, fax: (937) 255-1474 or e-mail: rebecca.clewell@wpafb.af.mil.

Rebecca A. Clewell
Operational Toxicology Branch

Attachments:

1. Double-labeled $^{36}Cl^{18}O_4^-$ study shows perchlorate is not metabolized *in vivo*
2. Analysis of cold perchlorate in rat thyroid by ion chromatography: ClO^{3-} and Cl^- do not interfere with ClO_4^- analysis
3. Perchlorate accumulation in tissues with NIS
4. Up-regulation of thyroid perchlorate with increased NIS expression
5. Radiolabeled perchlorate study suggests perchlorate is translocated into thyroid stroma, follicle and lumen
6. Kinetic behavior of perchlorate in rat thyroid suggests three-compartment model structure
7. Electrogenicity studies offer more than one explanation for reported results

8. Calculation of maximum thyroid perchlorate concentration assuming all measured perchlorate is bound to NIS in the thyroid membrane
9. Recent development in gestation model: Improved description of iodide uptake in the placenta
10. Comparison of perchlorate-specific parameters in PBPK models across life stages
11. References

3 April 2002

MEMORANDUM FOR EASTERN RESEARCH GROUP, INC.
ATTN: KATE SCHALK

This letter report has been coordinated at the branch level and is approved for release.

Richard R. Stotts, DVM, PhD
Branch Chief
Operational Toxicology Branch
Human Effectiveness Directorate

DOUBLE-LABELED $^{36}Cl^{18}O_4^-$ STUDY SHOWS PERCHLORATE IS NOT METABOLIZED *IN VIVO*

During the peer review meeting, reference was made to a double-labeled perchlorate ($^{36}Cl^{18}O_4^-$) study by Anbar *et al*. (1959) that was cited in the draft RfD document (page 3-3). The incomplete description of the study has led to the general concern that ClO_4^- may be metabolized as far as Cl^- before elimination in the urine. However, the contention of Anbar and coauthors is that ClO_4^- is **not** metabolized *in vivo*. The authors' comments regarding the results of their study are given below.

Amounts of Cl^- and ClO_3^- in the urine constitute "an upper limit for the metabolization of perchlorate ions, namely less than 1/1000 of the excreted perchlorate." Therefore, the maximum amount of perchlorate that could have been metabolized was less than 0.1% of the injected radiolabeled species.

Alternative reasons for detection of a small amount of chloride (Cl^-) and chlorate (ClO_3^-) were listed.

- The small amount of other chlorine species may have been due to contamination of the $K^{36}Cl^{18}O_4^-$ with less than 0.1% $K^{36}Cl^-$.

- Some perchlorate may have absorbed on the silver chloride precipitant used to measure chloride concentration.

Therefore, the authors assumed there was "practically no reduction of perchlorate ions *in vivo*" (Anbar *et al.*, 1959).

ANALYSIS OF COLD PERCHLORATE IN RAT THYROID BY ION CHROMATOGRAPHY: ClO^{3-} AND Cl^- DO NOT INTERFERE WITH ClO_4^- ANALYSIS

Our laboratory has measured serum and thyroid perchlorate levels in the rat and found that perchlorate is concentrated in thyroid tissue. In fact, the thyroid to serum (T:S) ratios were as high as 30:1 in the male rat after exposure to ClO_4^- in drinking water. Rats subjected to acute ClO_4^- exposures also showed T:B ratios greater than one. Table 1 shows the T:B ratios in adult male rats at various times after administration of 0.01, 1.0 and 3.0 mg ClO_4^-/kg body weight (BW).

Table 1. Thyroid:serum ratios after ClO_4^- *iv* administration in adult male rats

Time	0.01 mg/kg			1.0 mg/kg			3.0 mg/kg		
(hours)	thyroid	serum	T:S	thyroid	serum	T:S	thyroid	serum	T:S
0.08	0.3	0.028	**10.7**	20.26	1.21	**16.7**	23.28	3.12	**7.5**
0.25	0.3	0.023	**13.0**	22.21	1.12	**19.8**	31	3.31	**9.4**
0.5	0.36	0.025	**14.4**	20.99	1.29	**16.3**	29.78	3.16	**9.4**
1	0.25	0.024	**10.4**	22.01	0.98	**22.5**	34.57	3.17	**10.9**
2	0.26	0.022	**11.8**	24.2	0.78	**31.0**	33.22	2.95	**11.3**
6	0.26	0.018	**14.4**	14.76	0.4	**36.9**	17.85	0.91	**19.6**
9	0.09	ND		9.26	0.23	**40.3**	12.66	1.03	**12.3**
24	0.01	ND		5.56	0.21	**26.5**	10.76	0.54	**19.9**

ND – indicates samples that were below analytical detection

These thyroid samples were obtained from rats exposed to ClO_4^- either through drinking water or via tail vein injection. Tissues were then homogenized with water and filtered. The prepared thyroid tissue samples were analyzed for ClO_4^- using ion chromatography (IC) and a modified version of EPA Method 314, using a 100 mM NaOH mobile phase and an AS-11 column. This method utilizes both size and charge density to separate anions on an ion exchange column. Some ions, such as perchlorate, are tightly bound to the exchange medium in the column and therefore remain in the column for longer periods of time. Other anions, such as ClO_3^-, Cl^-, F^- and I^-, move quickly through the column. Thus, it is possible to distinguish between similar anions based on their retention times with confidence.

To rule out the possibility of interference by the potential metabolites of ClO_4^-, the IC method was tested against standards spiked with chlorate and perchlorate, as well as thyroid samples obtained from rats dosed with ClO_4^-. Thyroids were taken from two ClO_4^- exposed rats. One thyroid served as perchlorate-only control and the second thyroid sample was spiked with ClO_3^-. The resulting chromatograms are shown below (Figures 1 through 3).

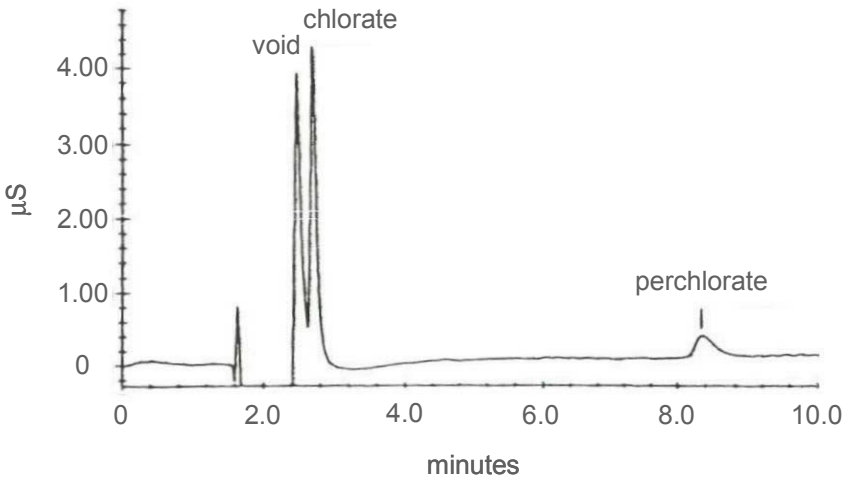

Figure 1. Water standard spiked with perchlorate and chlorate standards. The void peak appears immediately after the dip in the baseline due the water from the injected sample. Perchlorate is well separated from chlorate by approximately six minutes.

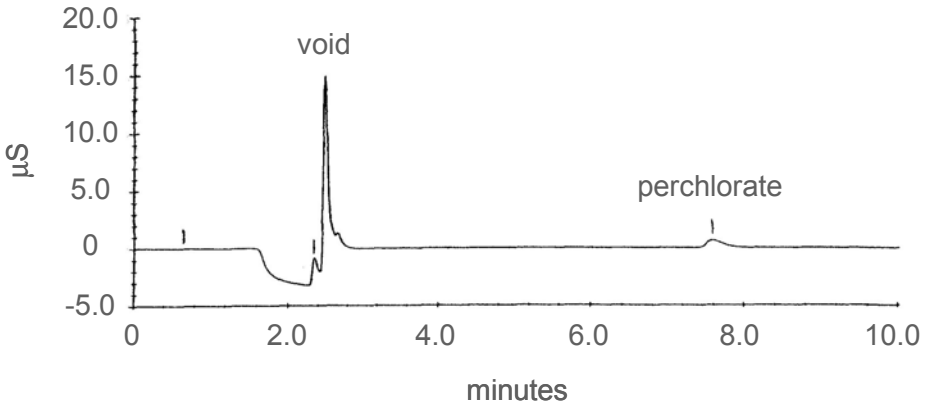

Figure 2. Typical chromatogram obtained from a thyroid of a rat exposed to perchlorate. The perchlorate peak appears at approximately eight minutes and is well separated from the initial void volume.

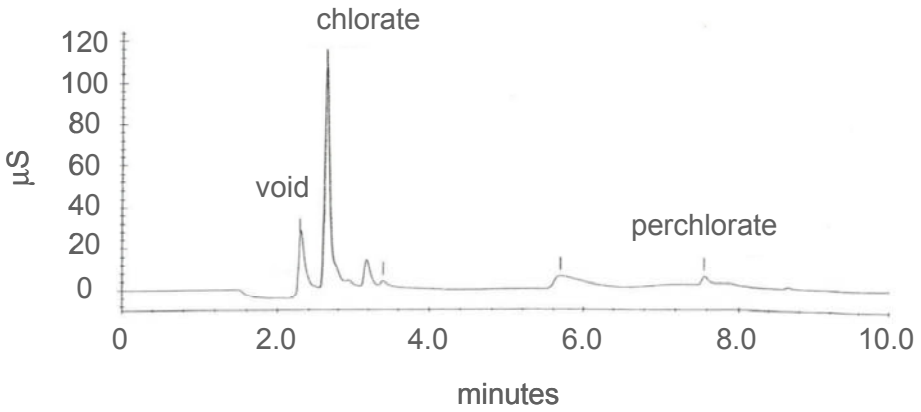

Figure 3. Chromatogram of a rat thyroid exposed to perchlorate *in vivo* and spiked with a chlorate standard after removal from the carcass. The chlorate peak appears shortly after the void peak and is well separated from the perchlorate peak.

In previous studies, this laboratory explored the relative affinity of several potential interfering anions for the ion exchange column, including ClO_3^- and Cl^- (Clewell, 1999). The method was identical to that used for the samples above, except that the concentration of the mobile phase was lowered slightly from 100 mM to 35 mM NaOH in order to better separate the earlier peaks. These studies showed that Cl^- had even less affinity for the AS-11 column than ClO_3^-. Thus, chloride could not be interfering with the ClO_4^- measurements.

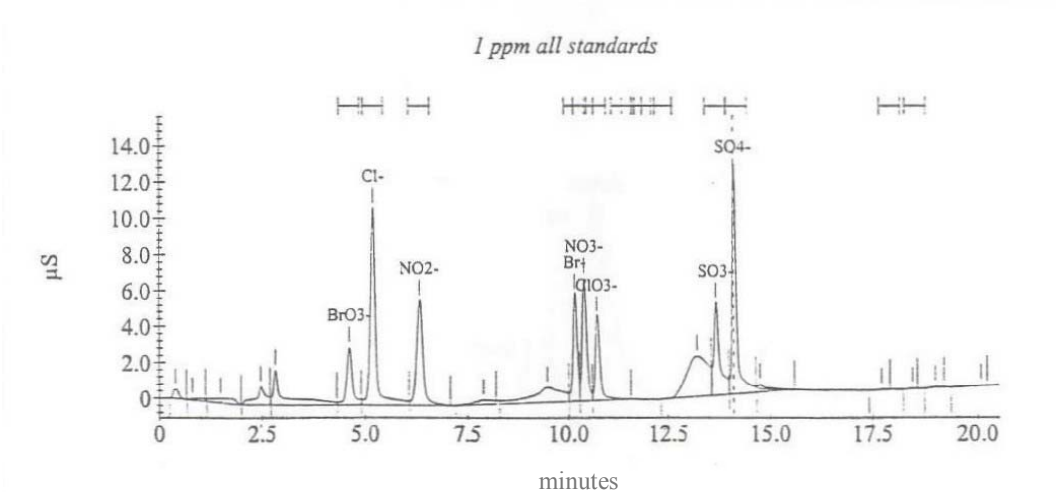

Figure 4. Chromatogram of water spiked with various anions, including chlorate and chloride. Chlorate has a higher affinity for the exchange column than chloride and therefore has a larger retention time. Perchlorate has a higher retention time than both potential metabolites and did not elute from the column within the 20 minutes.

It is apparent from the chromatograms shown above that the IC method is able to distinguish ClO_4^- from ClO_3^- and Cl^- without difficulty. The measured peaks in samples from ClO_4^- exposures correspond well with the retention time of the ClO_4^- standards. The perchlorate peak is well separated from interferences in the control thyroid samples and does not show any overlap with the peaks from the ClO_3^- and Cl^- standards.

PERCHLORATE ACCUMULATION IN TISSUES WITH NIS

ClO_4^- was consistently found to be concentrated in NIS-containing tissues. Intra-laboratory studies collected tissues known to express NIS after dosing Sprague-Dawley rats with ClO_4^- via drinking water and *iv* including, gastrointestinal (GI) contents, skin and milk from the lactating mammary gland. Tables 1 and 2 present data collected from the lactation drinking water ClO_4^- studies (Yu *et al.*, 2000b) used for development of the lactation model (Clewell *et al.*, 2001a). Female rats were exposed to ClO_4^- in drinking water from gestation day (GD) 2 to postnatal day (PND) 5 or 10. The tissue:serum ratios are consistently greater than one, suggesting active transfer into these tissues.

Table 1. Perchlorate in maternal tissues on PND 5

Dose Group (mg/kg-day)	Serum (µg/mL)		Thyroid (µg/mL)		Skin (µg/mL))		GI contents (µg/mL)	
	mean	SD	mean	SD	mean	SD	mean	SD
0.01	0.057	0.006	0.7	0.09	ND	ND	ND	ND
0.1	0.31	0.03	4.69	0.49	0.4	0.04	0.3	0.03
1	0.5	0.04	14.29	1.76	2.52	0.28	1.21	0.15
10	4.72	0.48	61.22	6.61	7.02	0.80	5.45	0.49

Table 2. Perchlorate in maternal tissues on PND 10

Dose Group (mg/kg-day)	Serum (µg/mL)		Milk (µg/mL)		Thyroid (µg/mL)	
	mean	SD	mean	SD	mean	SD
0.01	0.225	0.055	0.354	0.04	1.13	0.15
0.1	0.358	0.135	0.766	0.119	4.45	0.52
1	0.643	0.139	1.565	0.313	22.29	1.52
10	3.548	0.838	6.869	1.97	43.86	4.15

Active uptake of perchlorate by NIS-containing tissues was also evident in acute *iv* studies performed on male rats. Male rats given an *iv* dose of 0.01 mg/kg ClO_4^- were sacrificed one and two hours post-dosing and several of the tissues with NIS were analyzed for ClO_4^- levels. Measured serum, skin, stomach contents and thyroid ClO_4^- concentrations are shown in Table 3 (Yu *et al.*, 2000a).

Table 3. Perchlorate in male rat after 0.1 mg ClO_4^-/kg *iv* dose

Time (hours)	Skin (μg/g) [a]		Stomach Contents (μg/g) [a]		Thyroid (μg/g) [b]		Serum (μg/g) [a]	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
0.25					3.20	0.29	0.07	0.01
1	0.22	0.02	8.21	0.86	3.50	0.34	0.16	0.01
2	0.24	0.02	1.13	0.11			0.13	0.01

[a] Mean ± SD, n = 6

[b] Mean ± SD, n = 5 to 6

Tissues lacking NIS did not show the same accumulation of ClO_4^- as those tissues are known to contain the symporter. Tissue:serum ratios measured in muscle, fat and liver were less than one at all time points (Yu *et al.*, 2000a; Merrill *et al.*, 2001b). The fact that ClO_4^- is consistently sequestered only in tissues with NIS suggests that the symporter, and not another anion channel, is responsible for its uptake.

UP-REGULATION OF THYROID PERCHLORATE WITH INCREASED NIS EXPRESSION

Up-regulation of the iodide transfer into the thyroid via NIS is accomplished through an intricate feedback system, wherein decreased thyroid hormone levels in the serum signal the hypothalamus-pituitary-thyroid axis to produce thyroid stimulating hormone (TSH). This production of TSH causes an increase in the number and activity of NIS, resulting in increased transfer of iodide across the basolateral membrane into the thyroid follicle. In the PBPK models, this increased transfer of iodide across the thyroid basolateral membrane is represented by an increase in the follicular Vmax ($VmaxcT_i$). In rats exposed to ClO_4^- in drinking water for two weeks, increased uptake of iodide and ClO_4^- was seen in the up-regulated thyroids. In fact, as the thyroid goes from a naïve to a TSH-induced up-regulated state, the follicular Vmax for ClO_4^- ($VmaxcT_p$) increases proportionately with that of iodide. This strongly suggests that NIS, not another channel, is likely the main transporter of perchlorate.

An intra-laboratory study was performed, in which Sprague-Dawley rats were exposed to ClO_4^- in drinking water at doses of 0.01, 0.1, 1.0, 3.0, 10.0 and 30.0 mg ClO_4^-/kg-day for 14 days. On day 14, rats were challenged with an *iv* dose of 0.033 mg/kg $^{125}I^-$ (with carrier). TSH levels were increased in all groups receiving ClO_4^- within 24 hours and remained significantly higher than controls through day 14. However, a slight decrease was seen between days 5 and 14 (Yu *et al.*, 2000a). On day 14, no measurable inhibition of thyroid $^{125}I^-$ uptake was seen in the 0.01 through 10.0 mg/kg-day dose groups. Only the 30.0 mg/kg-day dose group showed slight inhibition after 2 weeks of ClO_4^- exposure. Thus, it can be concluded that thyroid was fully compensated (up-regulated) within the two exposure weeks for all but the highest dose group. We can assume that the increase in the iodide follicular Vmax [$VmaxTF_{i(upregulated)}$] exactly compensates for the ClO_4^- induced inhibition (represented in the model as an increase in the apparent follicular Km for iodide (KmTFi)). Equation 1 demonstrates that the overall uptake of an up-regulated thyroid is equal to that of a naïve thyroid:

$$\frac{V\,max\,TF_i \times CVTS_i}{KmTF_i + CVTS_i} = \frac{V\,max\,TF_{i(upregulated)} \times CVTS_i}{CVTS_i + KmTF_i\left(1 + {CVTS_p}/{KmTF_p}\right)} \qquad \text{Equation 1}$$

where:

$RupTF_i$ = Rate of active uptake of $^{125}I^-$ into the thyroid follicle (ng/hour)
$VmaxTF_i$ = maximum capacity of $^{125}I^-$ at the thyroid follicle (ie. NIS sites) (ng/hour)
$KmTF_i$, = Michaelis-Menten (M-M) affinity constant for $^{125}I^-$ at NIS (ng/L)
$CVTS_i$ = blood $^{125}I^-$ concentration in thyroid stroma (ng/L)
$KmTF_p$ = (M-M) affinity constant for ClO_4^- (competitive inhibitor) at NIS (ng/L)
$CVTS_{i,p}$ = $^{125}I^-$ or ClO_4^- concentration in thyroid stroma (ng/L).

By rearranging Equation 1, we find that the value for the up-regulated follicular Vmax can be calculated by the following:

$$VmaxTF_{i(upregulated)} = \frac{VmaxTF_{i(naive)} \times \left(CVTS_i + KmTF_i \left(1 + {CVTS_p}/{KmTF_p} \right) \right)}{CVTS_i + KmTF_i} \qquad \text{Equation 2}$$

Since the iodide concentration in the thyroid stroma ($CVTS_i$) is negligible in comparison to $KmTF_i$, it can be removed from the equation.

$$VmaxTF_{i(upregulated)} = VmaxTF_{i(naive)} \times \left(1 + {CVTS_p}/{KmTF_p} \right) \qquad \text{Equation 3}$$

If ClO_4^- is truly a competitive inhibitor, NIS would be responsible for transporting both anions into the follicle. Thus, one would expect a simultaneous increase in the follicular Vmaxs for both anions. The change in the $VmaxT_p$ should then be proportional to that of $VmaxT_i$, resulting in the up-regulated value [$VmaxTF_{p(upregulated)}$] shown below.

$$VmaxTF_{p(upregulated)} = \frac{VmaxTF_{i(upregulated)}}{VmaxTF_{i(naive)}} \times VmaxTF_{p(naive)} \qquad \text{Equation 4}$$

Figure 1 shows measured thyroid iodide uptake after 14 days of exposure to 0.01, 0.1, 1.0, 3.0, 10.0 and 30.0 mg ClO_4^-/kg-day versus simulations from the previous model (naïve $VmaxTF_i$) and using Equation 3 for up-regulation of $VmaxTF_i$.

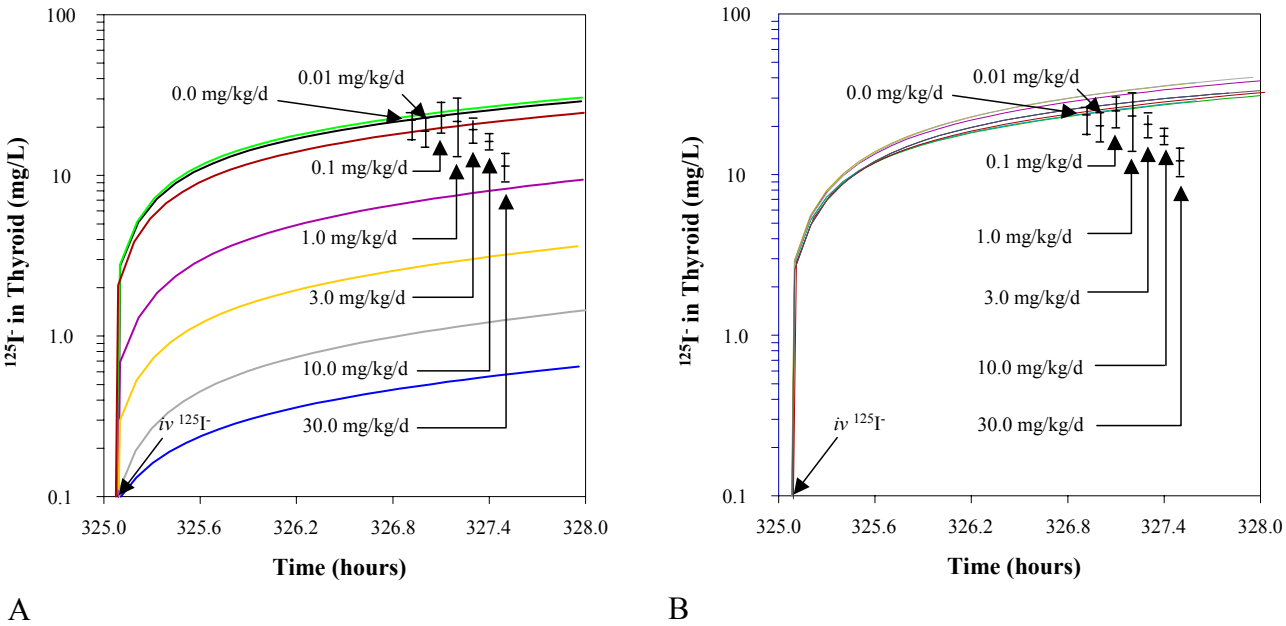

Figure 1. (A) Previous model over-prediction $^{125}I^-$ uptake inhibition in male rat thyroids after 14 days of ClO_4^- exposure in drinking water at 0, 0.01, 0.1, 1.0, 3.0, 10.0 and 30.0 mg/kg-day, followed by a 33 mg/kg $^{125}I^-$ *iv* with carrier. (B) Same prediction using Equation 3 to predict up-regulated $VmaxTF_i$. Solid lines and bars represent model prediction and mean ± SD of measured data, respectively.

Figure 2A shows the predictions of thyroid ClO_4^- concentrations from the previous model based on values from a naïve thyroid (Merrill *et al.*, 2001b). The model is able to simulate the data at the 1.0 mg/kg-day dose and below through day 14, and up to 10.0 mg/kg-day through day 1. The model was unable to reproduce the increased ClO_4^- uptake on day 5 and 14 in the 3.0, 10.0 and 30.0 mg/kg-day dose groups. Figure 2B shows model simulations of the same data using Equation 4 to calculate the up-regulated $VmaxTF_p$. The model slightly over-predicts thyroid ClO_4^- concentrations at 3.0 mg/kg-day dose and higher. However, the simulations are within the standard deviations of the day 14 data.

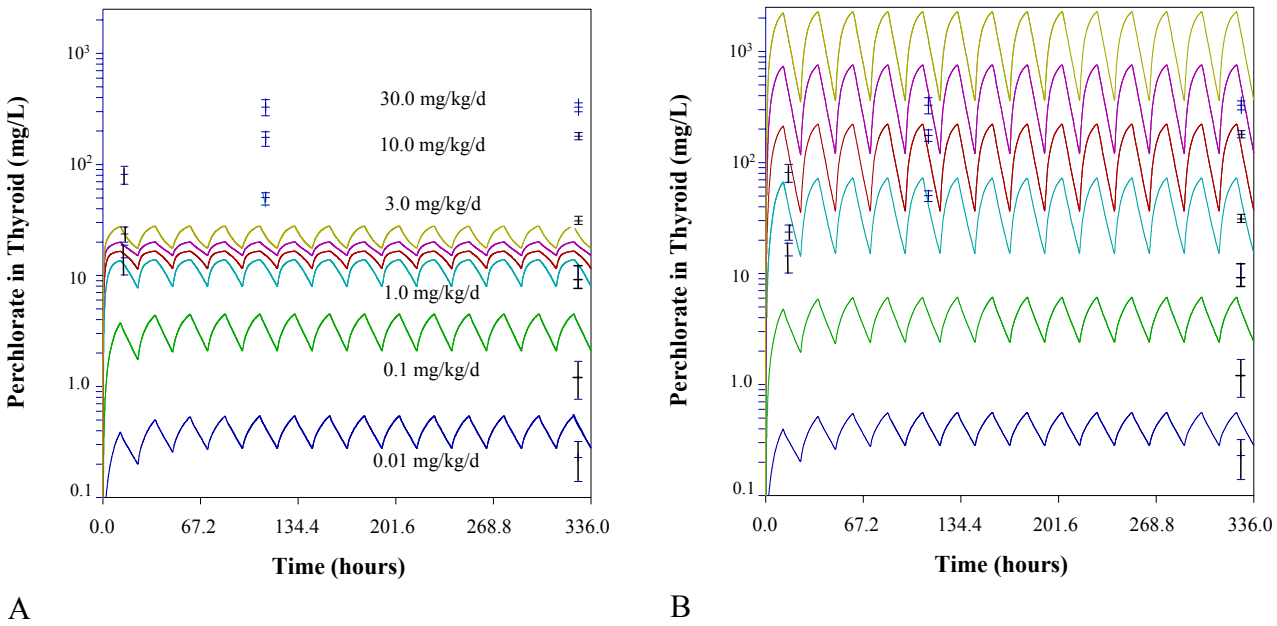

Figure 2. (A) Simulations using *VmaxTF$_p$* established from acute data fail to predict thyroid ClO_4^- concentrations at drinking water doses greater than 1.0 mg/kg-day. (B) Simulations fit data across doses using Equation 4 for up-regulated *VmaxTF$_p$*. Model predicted (lines) and measured means ± SD (bars) show thyroid ClO_4^-concentrations during ingestion of 0.01, 0.1, 1.0, 3.0, 10.0 and 30.0 mg/kg-d ClO_4^- in drinking water for 14 days.

In conclusion, the changes in follicular Vmax needed to simulate the increased ClO_4^- and I^- concentrations in the up-regulated rat thyroid were found to be proportional to one another. This proportional change in maximum capacities of both anions suggests that the increased TSH up-regulates the transfer of both iodide and ClO_4^- into the thyroid. Further evidence of TSH-induced up-regulation of thyroid ClO_4^- uptake is seen in the work of Chow *et al*. (1969) and Lewitus *et al*. (1962). Chow, Lewitus and their coauthors dosed rats with TSH and followed this dose with ClO_4^-. Both reported increased thyroid ClO_4^- uptake in the groups dosed with TSH compared the control groups. Together, these studies provide compelling evidence that NIS is the major transport route for ClO_4^- in the thyroid.

RADIOLABELED PERCHLORATE STUDY SUGGESTS PERCHLORATE IS TRANSLOCATED INTO THYROID STROMA, FOLLICLE AND LUMEN

Chow and Woodbury (1970) measured $^{36}ClO_4^-$ uptake in the thyroids of rats and guinea pigs after ip administration of the radiolabeled ion. Some of the animals were used to study time course of $^{36}ClO_4^-$ in the thyroid, while others were used to examine histological changes in the thyroid and changes in electrochemical potential in the presence of $^{36}ClO_4^-$.

The authors examined the $^{36}ClO_4^-$ time course data and observed that there were two apparent phases in the uptake of $^{36}ClO_4^-$ into the thyroid, the first being a rapid transport ($t_{1/2}$ = 2 min) and the second being a slower transport ($t_{1/2}$ = 33 min). The authors suggest that the first phase represents the rapid transport into the interstitial and cellular compartments. The second phase represents the rapid transport and slow equilibration of $^{36}ClO_4^-$ in the luminal fluid. The data, along with the histological measurements of the stroma, cell, and lumen, were used to calculate the relative $^{36}ClO_4^-$ concentrations of the compartments. This process was repeated for Cl^-. Chloride did not show the same accumulation in the thyroid seen with perchlorate. The values obtained for the relative compartment concentrations of the two anions are given below for two of the measured doses (Chow and Woodbury, 1970).

Table 1. Relative Concentrations of Perchlorate and Chloride in the Thyroid Compartments from Chow and Woodbury (1970).

$K^{36}ClO_4$ (mmol/kg)	Cell:Stroma		Lumen:Stroma		Lumen:Cell	
	$^{36}ClO_4^-$	$^{36}Cl^-$	$^{36}ClO_4^-$	$^{36}Cl^-$	$^{36}ClO_4^-$	$^{36}Cl^-$
0.005	6.15	0.14	147	0.95	24.0	6.8
0.01	2.2	0.13	48	1.0	21.6	7.7

From the time course behavior and measured potentials in the cellular compartments, the authors conclude, "$^{36}ClO_4^-$ is concentrated in the lumen of the thyroid by a two-step process. Firstly, $^{36}ClO_4^-$ is actively transported across the basal cell membrane from the stromal fluid into the cellular fluid. In the cellular fluid, $^{36}ClO_4^-$ then passes across the apical cell membrane into the lumen and it is further concentrated during this process" (Chow and Woodbury, 1970).

KINETIC BEHAVIOR OF PERCHLORATE IN RAT THYROID SUGGESTS THREE-COMPARTMENT MODEL STRUCTURE

A three compartmental thyroid, consistent with the physiological structure (i.e., stroma, follicle and lumen) is required to capture the kinetic behavior of thyroid ClO_4^- (Figure 1). This was also found to be true by Chow and Woodbury (1970). They found that the uptake curve for ClO_4^- showed two distinct phases, corresponding to a rapid transfer into the stroma and follicle and slower transfer into the lumen (see Attachment 7). Intra-laboratory data show similar results in the clearance of ClO_4^- in the thyroid of the male rat after *iv* dose of radiolabeled perchlorate. The ClO_4^- appears to be cleared from the thyroid in two phases. The first shows quick elimination from the follicle; the second is slower, suggesting slow release from a deep compartment (the thyroid lumen).

The models were originally described with two compartments, the stroma and cell. However, the two-compartment structure failed to simulate the behavior of ClO_4^-. The model could not simulate the 2 phases, thus resulting in a model that could either fit the first two data points or the last, but could not capture the kinetic behavior of the time course data. ClO_4^- was taken up and released from the follicle too quickly in the model (Figure 1). With a three-compartment model of the thyroid, it was possible to simulate the entire time course with one set of parameters. The kinetic behavior is well described assuming the rapid transfer between the stroma and follicle, and a slower release from the lumen.

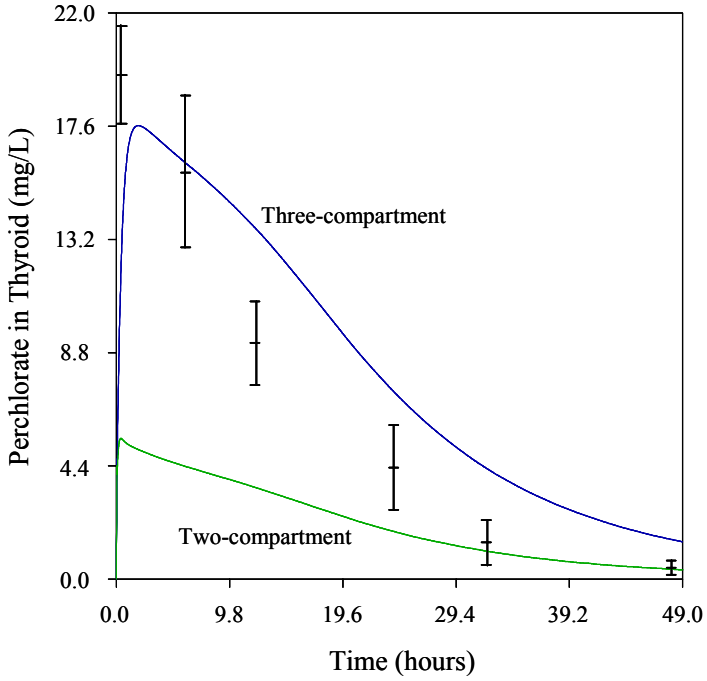

Figure 1. Thyroid $^{36}ClO_4^-$ concentration in the male rat after *iv* administration of 3.3 mg/kg with a two-compartment (bottom) and three-compartment (top) thyroid. Solid lines and bars represent the model simulation and mean ± SD of the measured data.

ELECTROGENICITY STUDIES OFFER MORE THAN ONE EXPLANATION FOR REPORTED RESULTS

The sodium-iodide symporter is a protein located on the basolateral membrane of thyroid follicular cells. NIS transports iodide into the thyroid follicle against a concentration gradient by way of an energy dependent co-transport mechanism (Na^+/K^+-ATPase). NIS transports two sodiums (Na^+) for every one iodide (I^-) and is driven by an inwardly directed Na^+ gradient. This 2:1 Na^+/I^- ratio has been established by several investigators (Kosugi *et al*., 1996; Eskandari *et al*., 1997; Riedel *et al*., 2000).

Recent studies on NIS electrogenicity have shown that a bathing medium containing I^- causes an inward current in an oocyte with NIS. When 500 μM ClO_4^- was added to the bathing medium, the inward current disappeared (Eskandari *et al*., 1997). Two possible reasons were given for the disappearance of the current.

1) Perchlorate blocks I^- transport, but may not be transferred into the thyroid cell.
2) Perchlorate competes with I^- and is transported by NIS at a 1:1 ratio with Na^+.

Although several authors (Riedel 2001a, b) have stated their preference for the first explanation, that ClO_4^- is not translocated into the cell, they all have also concluded that ClO_4^- transport by NIS at 1:1 stochiometric ratio with Na^+ is also a viable explanation (see also Nilsson, 1999; Dohan *et al*., 2000). Such a stochiometry would abolish the iodide-driven inward current but maintain the competitive inhibition of iodide uptake. These studies offer a compelling argument for the relationship of structure activity to the effectiveness of the thyroid uptake inhibitor. They also open the field to further research, in order to more clearly define the mechanism of these iodide uptake inhibitors. However, these studies do not attempt to quantitatively measure ClO_4^- in the thyroid, and they do not offer concrete evidence disproving the translocation of ClO_4^- across the follicular membrane. When these electrogenicity studies are taken in context with the many other studies in literature (Wolff, 1998; Chow and Woodbury, 1970; Hildebrandt and Halmi, 1981; *etc*.) and recent experiments (Yu *et al*., 2000a, b), the weight of evidence suggests that perchlorate is a truly competitive inhibitor. Thus, the mode of action, which serves as the foundation for the kinetic models, was based upon the entire body of available information.

CALCULATION OF MAXIMUM THYROID PERCHLORATE CONCENTRATION ASSUMING ALL MEASURED PERCHLORATE IS BOUND TO NIS IN THE THYROID MEMBRANE

One suggested mechanism by the authors of several articles (Eskandari *et al*., 1997; Riedel 2001a, b; Nilsson, 1999; Dohan *et al*., 2000) and by members of the Peer Review Panel, involves the binding but not translocation of ClO_4^- at the follicular membrane of the thyroid by NIS. In other words, the ClO_4^- anion is able to compete with iodide for binding sites on the NIS. However, when bound to ClO_4^- the NIS is not able to cross the follicular membrane. Thus, the ClO_4^- measured in the thyroid would have to be bound to the symporter in the membrane of the cell and not within the cell itself.

If true, this theory would require that all measured ClO_4^- molecules be bound to NIS in order to be found within the cellular membrane at the time of analysis. Thus, each perchlorate molecule would correspond to one inhibited symporter. This line of thought has two direct consequences.

- The relationship of iodide inhibition and perchlorate concentration should be linear.
- The ClO_4^- thyroid concentration that results in 100% inhibition should also be the maximum possible thyroid concentration.

In order to check the feasibility of this proposed mode of action, the measured ClO_4^- concentrations were plotted against the corresponding inhibitions in the thyroids of adult male rats given an *iv* dose of 0.01, 0.1, 1.0 and 3.0 mg ClO_4^-/kg BW (Yu *et al*., 2000a). Figures 1 and 2 show the relationship of ClO_4^- concentration to % inhibition in the rat thyroid. The non-linearity of this relationship is evident in both sets of measured values.

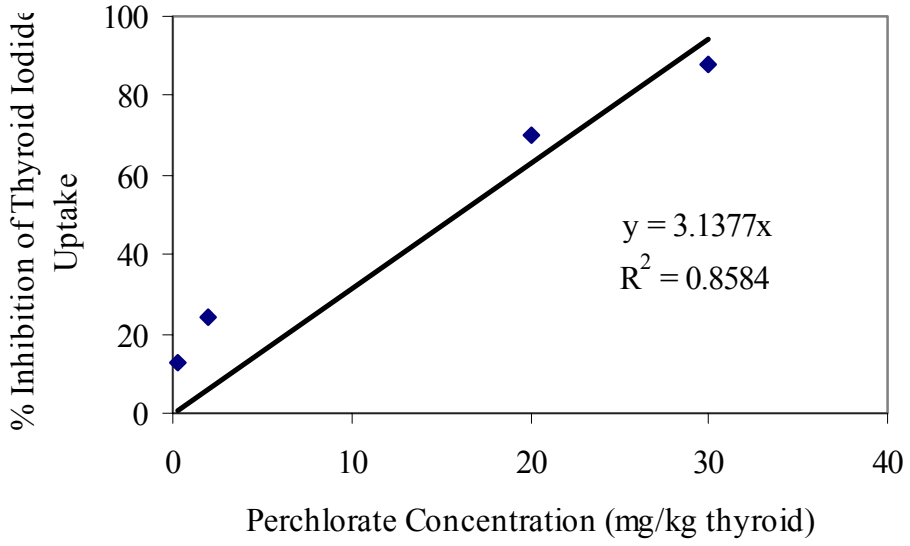

Figure 1. Plot of perchlorate concentration versus percent inhibition of iodide uptake in the thyroid at two hours post-dosing.

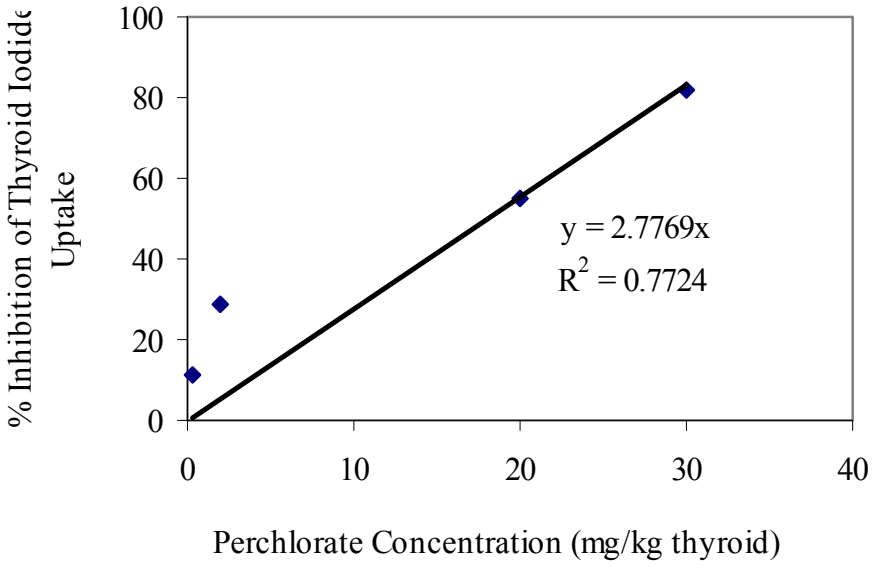

Figure 2. Plot of perchlorate concentration versus percent inhibition of iodide uptake in the thyroid at nine hours post-dosing.

In spite of the apparent non-linear dose-response curves, a straight line was fit to the measured data points and an equation was generated from the relationship of the thyroid ClO_4^-

concentration to the inhibition of iodide uptake. Again, if the above hypothesis is correct, the amount of ClO_4^- in the thyroid follicle should correspond to the number of unavailable NIS binding sites. Thus, at 100% inhibition, the thyroid ClO_4^- concentration should reach its maximum concentration. Setting y = 100 in the equations from Figure 1 and 2 yields maximum ClO_4^- concentrations in the male rat thyroid of 31.9 and 36.0 mg ClO_4^-/kg tissue, respectively.

In order to test the accuracy of the calculated maximum thyroid ClO_4^- concentration, measurements were needed in the thyroid of a non-upregulated rat after *in vivo* ClO_4^- exposure. These data were not available above a dose of 3.0 mg/kg BW within this laboratory. However, Chow and Woodbury (1970) measured the thyroid ClO_4^- concentration in the male rat after an *iv* dose of 14 mg ClO_4^-/kg BW and found ClO_4^- concentrations of 270 mg/kg-thyroid at this dose.

Neither of the criterion for this proposed mode of action are satisfied when tested against data. The non-linearity of the dose-response curve suggests that the thyroid ClO_4^- concentration is not directly related to the iodide inhibition. In the lower doses, there is apparently more ClO_4^- in the thyroid than can be accounted for by occupied NIS. The calculated maximum ClO_4^- concentration also underestimates the ability of the thyroid to accumulate ClO_4^-. The fact that 100% occupancy of the NIS cannot account for the mass of ClO_4^- measured in the thyroid indicates that the ClO_4^- ion must also be located in other areas of the thyroid, not exclusively in the membrane NIS. Thus, there must be translocation of the ClO_4^- anion in the thyroid cells.

RECENT DEVELOPMENT IN GESTATION MODEL: IMPROVED DESCRIPTION OF IODIDE UPTAKE IN THE PLACENTA

The PBPK models presented in the EPA document represent the progress in model development as of June 2001. However, since the submittal deadline, new data were made available, including measurements of perchlorate-induced inhibition of uptake in the placenta as well as several fetal tissues. The new data sets facilitated changes in the gestation and lactation models. Active uptake of iodide and ClO_4^- by NIS inhibition of placental iodide transfer by ClO_4^- was added to the gestation model. The presence of NIS in the placenta is supported by literature, as is the ability of ClO_4^- to reduce iodide levels in the fetus (Versloot *et al*., 1997). Due to time limitations and the lack of data, placental iodide and ClO_4^- transfer were reasonably described as first order clearance processes in the first version of the model (Clewell *et al.*, 2001b). However, further investigation allowed a more thorough model description of placental inhibition, which is vital when determining the ability of the fetal thyroid to obtain the iodide necessary for hormone synthesis.

This report will present the current fits of the model to the new data. A manuscript with the complete description of the gestation model is currently in preparation. The updated dosimetrics have been provided to the EPA and the external peer review panel in a previous consultative letter (Merrill *et al*., 2002). These changes resulted in significantly different values for fetal inhibition. However, they did not affect the values for the serum perchlorate concentrations used by the U.S. EPA for species/life stage extrapolation (USEPA, 2002). Therefore, these changes are provided, not to change the risk assessment, but to inform the peer review panel of further progress and to address their concern as to the description of the placenta.

New Experiments

Timed-pregnant Sprague-Dawley dams were given a tail vein injection of 1.0 mg ClO_4^-/kg BW on GD 20. Two hours post-dosing with ClO_4^-, dams were given a tracer dose (average dose = 1.87 ng/kg BW) of $^{125}I^-$ on GD 20. Dams (n=6) were sacrificed at 0.5, 1, 2, 4, 8, 12 and 24 hours post-$^{125}I^-$ dosing. Maternal and fetal serum, skin and GI tract, as well as maternal thyroid, GI content, placenta and mammary gland tissue were collected and analyzed for $^{125}I^-$ with a gamma counter. Fetal serum was pooled by litter due to small sample volume. Fetal skin and GI tract were analyzed individually. Inhibition kinetics were examined in this same manner. However, two hours prior to the administration of $^{125}I^-$ (average dose = 1.87 ng/kg BW), dams from the inhibition group were dosed with 1.0 mg/kg BW ClO_4^- via tail vein injection and euthanization was performed at 0.5, 1, 2, 4, 8, 12 and 24 hours post-$^{131}I^-$

Modifications to Model Structure

Active uptake was simulated in the placenta using Michealis-Menten kinetics as described for the other NIS compartments in the previous consultative letter (Clewell *et al.*, 2001b) and the EPA risk assessment document. Inhibition was added to the description of the placenta, maternal and fetal GI contents and skin in the manner described for the thyroid.

Figure 1 shows the new model schematic. Table 1 contains the revised perchlorate and iodide parameters. The only change from that of Clewell *et al.* (2001b) is the active uptake in the placenta compartment. Figure 2 shows the fit of the model to inhibition data in the maternal and fetal tissues. Table 3 shows the model prediction of inhibition of total fetal iodide measured by Sztanyik and Turai (1988). The model is able to adequately describe inhibition kinetics in the maternal and fetal rat and is able to predict inhibition of iodide transfer to the fetus with these modifications to the original structure.

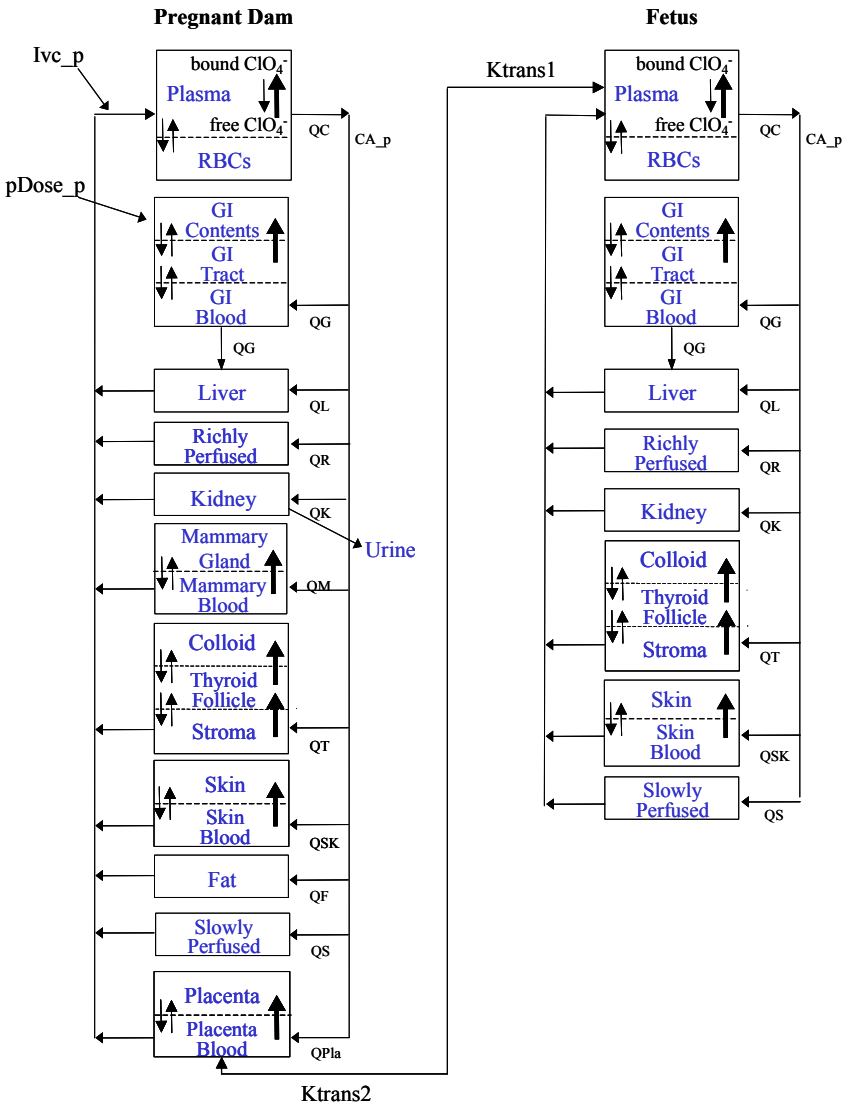

Figure 1. Schematic of PBPK model for pregnant dam (left) and fetus (right).

Table 1. Chemical Specific Parameters

Parameters	**Perchlorate**		**Iodide**	
Partition Coefficients (unitless)	**Dam**	**Fetus**	**Dam**	**Fetus**
Slowly Perfused / Plasma PS	0.31	0.31	0.21	0.21
Rapidly Perfused / Plasma PR	0.56	0.56	0.40	0.40
Fat/ Plasma PF	0.05	---	0.05	---
Kidney/ Plasma PK	0.99	0.99	1.09	1.09
Liver/Plasma PL	0.56	0.56	0.44	0.44
Gastric Tissue/Gastric Blood PG	0.50	1.80	1.00	1.00
Gastric Juice/Gastric Tissue PGJ	1.30	2.30	2.00	2.00
Skin Tissue/Skin Blood PSk	1.15	1.15	0.70	0.70
Thyroid Tissue/Thyroid Blood PTF	0.15/2.25[a]	0.15/2.26[a]	0.15	0.15
Thyroid Lumen/Thyroid Tissue PTL	7.0	7.0	7.0	7.0
Red Blood Cells/Plasma PRBC	0.73	0.73	1.00	1.00
Placenta/ plasma PPl	0.56	---	0.40	---
Mammary/Plasma PM	0.66	---	0.66	---
Max Capacity, Vmaxc (ng/hour/kg)				
Thyroid Follicle VmaxcTF	$1.8x10^3$	$0 – 2.25x10^3$	$4.0x10^4$	$0 –5.0x10^4$
Thyroid Colloid VmaxcTL	$1.0x10^4$	$1.0x10^{4b}$	$6.0x10^7$	$6.0x10^{7b}$
Skin VmaxcSk	$6.0x10^5$	$4.0x10^5$	$6.0x10^4$	$3.0x10^5$
Gut VmaxcG	$8.0x10^5$	$1.0x10^5$	$1.0x10^6$	$2.0x10^6$
Mammary VmaxcM	$2.2x10^4$	---	$5.0x10^4$	---
Placenta VmaxcP	$6.0x10^4$	---	$5.5x10^4$	---
Affinity Constants, Km (ng/L)				
Thyroid Follicle KmTF	$1.0x10^5$	$1.0x10^5$	$4.0x10^6$	$4.0x10^6$
Thyroid Colloid KmTL	$1.0x10^8$	$1.0x10^8$	$1.0x10^9$	$1.0x10^9$
Skin KmSk	$1.0x10^5$	$1.0x10^5$	$4.0x10^6$	$4.0x10^6$
Gut KmG	$1.0x10^5$	$1.0x10^5$	$4.0x10^6$	$4.0x10^6$
Mammary KmM	$1.0x10^5$	---	$4.0x10^6$	---
Placenta KmP	$1.0x10^5$	---	$4.0x10^6$	---
Permeability Area Cross Products, (L/hour-kg)				
Gastric Blood to Gastric Tissue PAGc	1.00	1.00	0.80	0.10
Gastric Tissue to Gastric Juice PAGJc	1.00	1.00	0.60	0.30
Thyroid Stroma to Follicle PATFc	$6.0x10^{-4}$/ $1.4x10^{-4}$	$6.0x10^{-4}$/ $1.4x10^{-4b}$	$1.0x10^{-4}$	$1.0x10^{-4}$
Thyroid Follicle to Colloid PATLc	0.01	0.01[b]	$1.0x10^{-4}$	$1.0x10^{-4}$
Skin Blood to Skin Tissue PASkc	1.00	1.00	0.10	0.02
Mammary Blood to Mammary Tissue PAMc	0.04	---	0.01	---
Placenta Blood to Placenta Tissue PAPc	0.1	---	0.005	---
Plasma to Red Blood Cells PARBCc	1.00	1.00	1.00	1.00
Clearance Values, (L/hour-kg)				
Urinary excretion ClUc	0.07	---	0.03	---
Transfer from Placenta to Fetus ClTrans1c	0.065		0.06	
Transfer from Fetus to Placenta ClTrans2c	0.12		0.12	
Binding Constants				
Association to Binding Sites VmaxcB	$4.0x10^3$	$1.5x10^3$	---	---
Affinity for Binding Sites KmB	$1.0x10^4$	$1.5x10^4$	---	---
Dissociation from Plasma Binding Sites ClUnbc	0.034	0.01	---	---

Notes: [a] Indicates values for up-regulated thyroid; [b] Scaled from adult value

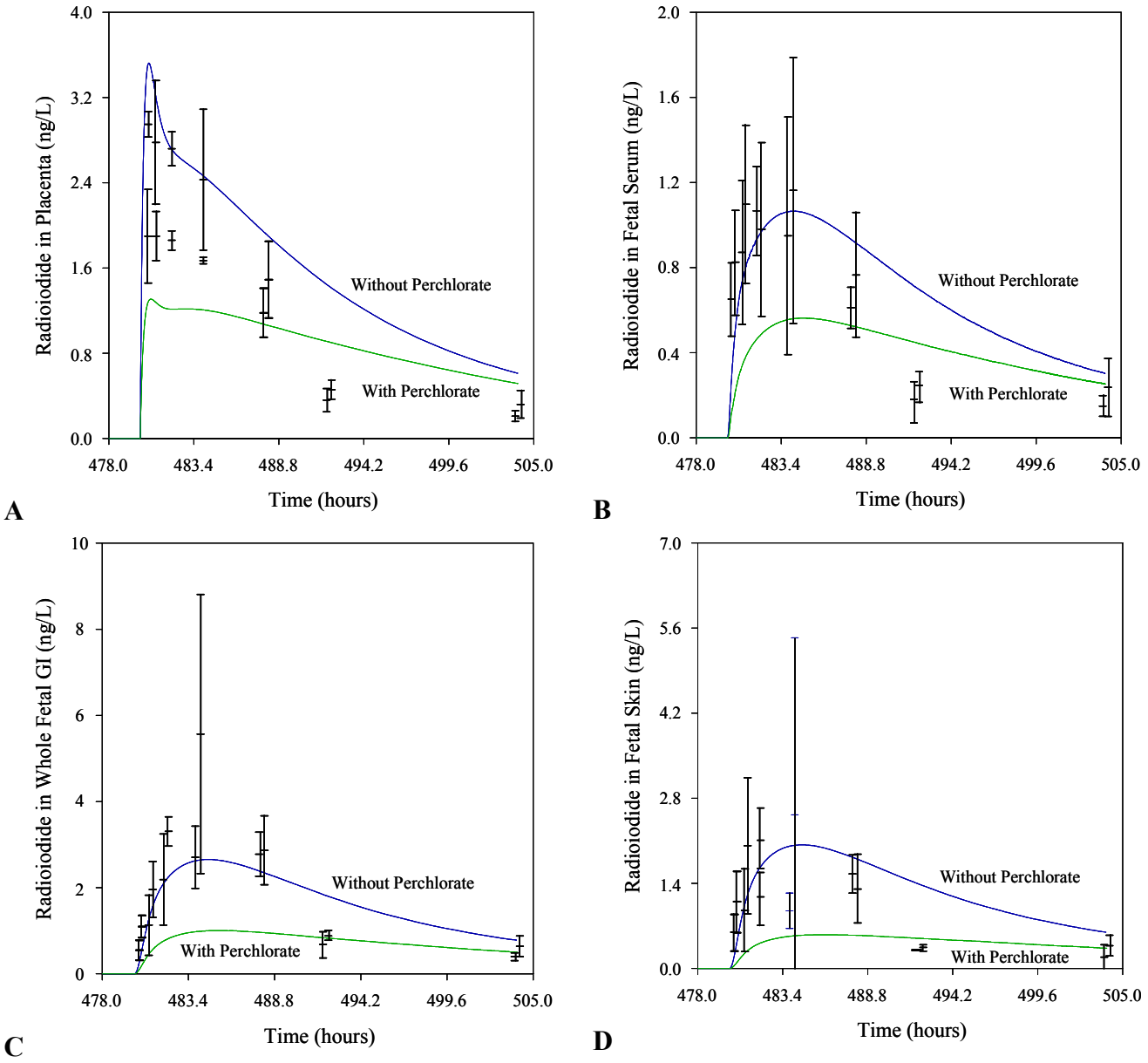

Figure 2. Radioiodide ($^{125}I^-$) concentration with and without 1.0 mg/kg administration in (A) placenta, and fetal (B) serum, (C) GI contents and (D) skin. Solid lines represent model predictions. Bars represent the mean ± SD of the data. Maternal GI contents and skin were not significantly different between control and inhibited animals in the data or model simulations.

Table 2. Model Predicted Inhibition of Iodide Uptake in Total Fetus Versus Data of Sztanyik and Turai (1988)

$KClO_4$ Dose (mg)	Time of ClO_4^- dose (after $^{131}I^-$ dose) (min)	Model Predicted % Inhibition in Fetus (% Control)	Measured % Inhibition in Fetus (% Control)
6.0	20	13%	8.1-15.1 (12)
3.0	60	18%	14.2-19.8 (17)
3.0	120	20%	24.2-34.6 (30)

COMPARISON OF PERCHLORATE-SPECIFIC PARAMETERS IN PBPK MODELS ACROSS LIFE STAGES

The perchlorate chemical specific parameters are nearly identical for the rat models across life stages, with the exception of the maximum uptake (*Vmaxc*) of ClO_4^- into the gastric juice/GI contents and the permeability area cross product (PA) in the thyroid follicle. Due the available data, the value $VmaxcG_p$ represents the saturable uptake in the gastric (stomach) juice of the male rat and human and the entire gastro-intestinal (GI) contents in the pregnant and lactating rats. Therefore, the values of these parameters cannot be directly compared between the male and developmental models.

The PA value for ClO_4^- uptake in the thyroid follicle is somewhat higher in the pregnant rat than the male rat. This difference is supported by the ClO_4^- time course data in both male and pregnant rats and by the fact that greater loss of radioiodide is also seen in during pregnancy in the rat in historical studies (Versloot *et al*., 1997). This loss is represented in the models by an increased value for PA at the follicular membrane (PATc), allowing the ion to move out of the cells more quickly.

Table 1. Comparison of Rat Model Perchlorate Parameters Across Life Stages

Perchlorate Parameters	Male Rat	Pregnancy		Lactation	
		Dam	Fetus	Dam	Neonate
Partition Coefficients (unitless)					
Slowly Perfused / Plasma PS_	0.31	0.31	0.31	0.31	0.31
Rapidly Perfused / Plasma PR_	0.56	0.56	0.56	0.56	0.56
Fat/ Plasma PF_	0.05	0.05	---	0.05	0.05
Kidney/ Plasma PK_	0.99	0.99	0.99	0.99	0.99
Liver/Plasma PL_	0.56	0.56	0.56	0.56	0.56
Gastric Tissue/Gastric Blood PG_	0.70	0.50	1.80	1.80	3.20
Gastric Juice/Gastric Tissue PGJ_	1.70	1.30	2.30	2.30	5.60
Skin Tissue/Skin Blood PSk_	1.15	1.15	1.15	1.15	1.15
Thyroid Tissue/Thyroid Blood PT_	0.13	0.13	0.13	0.13	0.13
Thyroid Lumen/Thyroid Tissue PDT_	7.00	7.0	7.0	7.0	7.0
Red Blood Cells/Plasma	0.80	0.73	0.73	0.73	0.73
Maximum Capacities (ng/hour/kg BW)					
Thyroid Follicle Vmaxc_T	2.2E+03	1.8E+03	0 - 2.25e3	1.5E+03	1.5E+03
Thyroid Colloid Vmaxc_DT	1.0E+04	1.0E+04	1.0E+04	1.0E+04	1.0E+04
Skin Vmaxc_S	6.2E+05	6.0E+05	4.0E+05	8.0E+05	8.0E+05
Gut Vmaxc_G	2.0E+05	8.0E+05	1.0E+05	1.0E+06	1.0E+06
Affinity Constants (ng/L)					
Thyroid Follicle Km_T	1.8E+05	1.0E+05	1.0E+05	1.0E+05	1.0E+05
Thyroid Colloid Km_DT	1.0E+08	1.0E+08	1.0E+08	1.0E+08	1.0E+08
Skin Km_S	2.0E+05	1.0E+05	1.0E+05	1.0E+05	1.0E+05
Gut Km_G	2.0E+05	1.0E+05	1.0E+05	1.0E+05	1.0E+05
Permeability Area Cross Products (L/hour/kg BW)					
Gastric Blood to Gastric Tissue PAGc_	1.00	1.00	1.00	1.00	1.00
Gastric Tissue to Gastric Juice PAGJc_	0.80	1.00	1.00	1.00	1.00
Thyroid Blood to Thyroid Tissue PATc_	4.0E-05	1.4E-04	1.4E-04	4.0E-05	1.0E-03
Thyroid Tissue to Thyroid Lumen PADTc_	0.01	0.01	0.01	0.01	1.0E-03
Skin Blood to Skin Tissue PASkc_	0.80	1.00	1.00	0.50	1.00
Plasma to Red Blood Cells PARBCc_	1.00	1.00	1.00	1.00	1.00
Clearance Values (L/hour/kg BW)					
Urinary excretion CLUc_	0.07	0.07	---	0.07	0.0075
Transfer from Placenta to Fetus	---	0.065		---	---
Transfer from Fetus to Placenta	---	0.120		---	---
Serum Binding Constants					
Vmaxc_Bp (ng/hour/kg BW)	3.5E+03	5.0E+03	1.5E+03	9.0E+03	2.0E+03
Km_Bp (ng/L)	1.1E+04	1.0E+04	1.5E+04	1.0E+04	1.0E+04
Kunbc_p (L/hour/kg BW)	0.028	0.034	0.034	0.034	0.01

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