Herrn Prof. Philipp Christen
Editorial Office, Eur. J. Biochem.
Apollostr. 2
Postfach A 152

CH-8032 Zürich
Switzerland

May 18, 1995

Manuscript: Transport and ATP synthesis in mitochondria (part 1 to 4)

Dear Prof. Christen,

I like to submit the original manuscript with the topic

"Transport and ATP synthesis in mitochondria" (part I to IV)

for publication in Eur.J.Biochem. The manuscript has been reviewed in the last years by

1) Cellular Physiol. and Biochem. (II.part)
2) FEBS letters (I. and II.)
3) Eur.J.Biochem. (I. - III.)
4) BBA (I. - IV.)
5) Biol. Chem. H.-S. (I. - IV.)
6) JMM (I. - IV.), which receives a review (under the heading: Glutathione, the essential factor for mitochondrial energy linked functions, etc.)
7) Chemistry-A.Eur.J. (I. - IV.) suggested to submit the manuscript to Eur.J.Biochem.
8) Biol.Chem.H.-S. (I. - IV.) means the manuscript unfortunately cannot be published in this journal.

The manuscript has been corrected under consideration of all the comments obtained by the various journals and it is not under consideration for publication elsewhere.

Sincerely

(Reinhold Kiehl)

Encl. Original Manuscript plus 3 Copies

European Journal of Biochemistry

Zürich, 23/05/95

Dr. Reinhold Kiehl
Laboratory and Research for
Mol. Medicine/Biology
Amselweg 12
D-93437 FURTH IM WALD
Germany

Reference no.: 95-0810 95-811 95-812

Transport and ATP synthesis in mitochondria
I. Evidence for mitochondrial 2,4-dinitrophenol accumulation across the Pi/H+-symport system

by

Kiehl Reinhold,

Editor: Pettersson

Dear Dr. Kiehl,

Thank you for submitting your manuscript.

Manuscripts cannot be published in the Journal without a specific statement from the authors that the work is not being, and has not been, published elsewhere, and that all authors approve its submission.

Please sign the enclosed copyright form and the computer questionnaire and return them by regular mail to the Editorial Office.

You will be advised of the Editor's decision in due course.

Yours sincerely,

L.H. Rowett

Dr. Lewis Rowett
Editorial Manager

Encl.: Copyright form
Computer questionnaire

Editorial Office: Apollostrasse 2, Postfach
CH-8032 Zürich, Switzerland

NEW TELEPHONE & FAX NUMBERS:
TEL. +41 (1) 388 20 40
FAX +41 (1) 388 20 41

European Journal of Biochemistry

Zürich, 25/07/95

Dr. Reinhold Kiehl
Laboratory and Research for
Mol. Medicine/Biology
Amselweg 12
D-93437 FURTH IM WALD
Germany

Reference no.: 95-0810 95-0811 95-0812

Transport and ATP synthesis in mitochondria
I. Evidence for mitochondrial 2,4-dinitrophenol accumulation across the Pi/H+-symport system

by

Kiehl Reinhold

Editor: Pettersson

Dear Dr. Kiehl,

Thank you for submitting your manuscript.

I regret to inform you that the Editor responsible for the manuscript has advised me that it cannot be accepted for publication in the Journal. The referees' reports are enclosed for your information.

The top copy of your manuscript will be returned to you by separate printed-matter mail.

Yours sincerely,

L.H. Rowett

Philipp Christen

Chairman of the
Editorial Board

Encl.: 2 reports
Copy : Editor

Editorial Office: Apollostrasse 2, Postfach
CH-8032 Zürich, Switzerland
Tel.:
Fax:

NEW TELEPHONE & FAX NUMBERS:
TEL. +41 (1) 388 20 40
FAX +41 (1) 388 20 41

95.081OP Report 1

The author claims that 2, 4-dinitrophenol is accumulated by the P_/H _ ,mporter, basing this on a number of highly unconvincing and inconsistent observations. The author uses the SH-reagent NSPM which is obviously much less specific for the phosphate carrier than for example mersalyl.

In Figure 1 quite confusing experimental results are given. With increasing concentrations of NSPM this swelling is increased, and the phosphate and calcium contents are decreased. What would be the osmotic reason for the swelling if the ion uptake is decreased?

It is actually doubtful that swelling is measured because 750 nm is an unusual wave length to measure mitochondrial ability. Further, what may have been observed is breakage of mitochondria. turbiding

Concerning the uptake of ^{14}C-DNP (Table 1) the unusual high concentration used of nearly 100 µm DNP may even inhibit the respiratory activity. The main conclusion of the paper reflected by the results in Table 1 are very doubtful. DNP at this high concentration could also dissolve in the lipid membrane of the mitochondria. The inhibition by NSPM does not give any evidence that the phosphate carrier is involved.

In summary, the paper contains few experiments which are performed under not very logical conditions. The conclusion that the phosphate carrier accumulates DNP is not at all born out by the actual experiments.

which phosphate carrier ??

EJB MS No. 95.081OP Report No.: 2 1/3

This manuscript by Kichi Reinhold presents measurements of N'-[N'-n-nonyl-4-sulfamoylphenyl]-maleimide (NSPM)-dependent inhibition of [^{14}C]DNP and ^{33}Pi accumulation into mitochondria and competition with the DNP analog, 2-azido-4-nitrophenol. The authors conclude that NSPM is a specific inhibitor of the phosphate binding site of the Pi / H$^+$-symport system and that 2,4-dinitrophenol (DNP) is transported into the mitochondria by this transporter.

If I understand the experiments presented in this manuscript, they do not warrant the strong conclusions presented by the author. It seems to me that, in spite of the authors claims, NSPM is a relatively non-selective inhibitor affecting various mitochondrial transporters and that the evidence for modification of the Pi / H transporter is based solely on the comparative inhibitor sensitivity of [^{14}C]DNP and Pi accumulated into the mitochondria. The authors second conclusion, that NSPM is a specific inhibitor of the phosphate binding site of the Pi / H$^+$-symport system is based on the NSPM-selective modification of the binding of a 2,4-dinitrophenol analog, 2-azido-4-nitrophenol. There are no controls to indicate that the photoactive inhibitor is competitive with DNP or Pi and this omission is exacerbated by the omission of experimental details and data analysis essential for evaluation.

I think a recurring difficulty in each manuscript of this series is the tendency to interpret complex phenomena in terms of specific proteins while failing to provide sufficient experimental detail to establish a unique mechanism of action. If I misunderstand these data it is largely due to the authors awkward writing style where necessary experimental detail is often absent, important points are not articulated and the discussion often rambles. Part of the problem may be due to a lack of familiarity with English, but in addition to complete editorial revision, major changes are needed to adequately describe and discuss the data within this manuscript. I have provided some specific comments for the author, but this is not an inclusive list.

Well/solid is an important point? relative !!

Explicit

95.081 ? Report 2

Comments to the author:

1. If I understand the experiments presented in this manuscript, they do not warrant the strong conclusions presented by the author. It seems to me that, in spite of the authors claims, NSPM is a relatively non-selective inhibitor affecting various mitochondrial transporters and that the evidence for modification of the Pi / H transporter is based solely on the comparative inhibiter sensitivity of [^{14}C]DNP and Pi accumulated into the mitochondria. The authors second conclusion, that NSPM is a specific inhibitor of the phosphate binding site of the Pi / H$^+$-symport system is based on the NSPM-selective modification of the binding of a 2,4-dinitrophenol analog, 2-azido-4-nitrophenol. There are no controls to indicate that the photoactive inhibitor is competitive with DNP or Pi and this omission is exacerbated by the omission of experimental details and data analysis essential for evaluation.

2. A recurring difficulty in each manuscript of this series is the tendency to interpret complex phenomena in terms of specific proteins while failing to provide sufficient experimental detail to establish a unique mechanism of action. The authors writing style is awkward where necessary experimental detail is often absent, important points are not articulated and the discussion often rambles. Part of the problem may be due to a lack of familiarity with English where thorough editorial revision is essential. In addition, each article within a series must be self-contained. If reference to another manuscript is essential, then the data from 1 should be included in that manuscript. Some combination of these manuscripts is desirable.

3. Please clarify the description of the experiment presented in fig 1.

As I understand this experiment, the difference between the protocols for the experiments of Panel A and Panel B is that in Panel A the mitochondria are equilibrated in the transport buffer (with ^{33}Pi) for 2 min., NSPM is added and the sample equilibrated for an additional 2 min. while in panel B the mitochondria are equilibrated in the transport buffer (minus ^{33}Pi) with NSPM for 2 min., ^{33}Pi is added and the sample equilibrated for an additional 2 min.

If this is the case, why is less ^{33}Pi accumulated following NSPM modification in the experiment of Panel A than that accumulated in the control in Panel B ? In other words, why wouldn't the ^{33}Pi accumulated in the untreated experiment in panel B represent the lower limit of the ^{33}Pi retained in Panel A at full inhibition ?

Additional data is needed in fig 1 to evaluate the quantitative relationship between the NSPM concentration dependence and the inhibition of ^{33}Pi accumulation into the mitochondria. A revised experiment should include at least 4 points within the concentration range of greatest effect (i.e 20 to 80% inhibition) as well as several points at near full inhibition.

4. The sentence structure throughout the manuscript is awkward and will require extensive revision to clarify the authors intent. Some representative examples are:

p. 5 line 20. The sentence beginning "As can be seen ..." is garbled. What relationship to which of the three activities are the authors referring ?

p. 6 lines 19-23 The sentence beginning " We titrated mitochondria..." is confusing because it combines at least 2 subjects. State succinctly what detergent concentration uncouples oxid. phos. and what detergent concentration inhibits the State 4 to State 3 transition.

Explicit

95.08108 Report 2

(7) p. 7 lines 6-9 The sentence beginning "20 nmoles NSPM/mg..." provides an incoherent description of the data of Table I

p.7 lines 11-14 The rationale for the experimental variation of Mg^{2+} to investigate the relationship between NSPM-dependent inhibition of DNP uncoupling (?) and accumulation is obscure. How does Mg^{2+} affect these parameters ? Was Mg^{2+} a variable in these measurements. This data, if relevant, should be organized into a Table or a figure.

(9) p. 7 line 18-22 The superficial introduction of results from other manuscripts does not strengthen this manuscript. If this data is important, present it . Otherwise, remove it. Provide references for the specificity of a particular inhibitor such as Cd^{2+} in the mitochondria.

(10) The legend to figure 2 and the experimental description in the Results do not provide sufficient detail to understand the data of this experiment. Describe this experiment and define the plot-derived kinetic constants. Provide controls that define which component of 2-azido-4-nitrophenol bound to the mitochondria is sensitive to DNP and Pi. While the mechanism of inhibition may provide important clues to the site of an inhibitor, this information is of an indirect structural nature. It is important to recognize the limitations of this data.

5. The Discussion frequently sites data within other manuscripts, but provides a minimal discussion of their own data. For example:

(11) 1. The statement on p 9 lines 2-4 concludes that Pi, DNP and NPA are bound and transported at the expense of the proton gradient. Since binding and transport are not measured in each, the model system is complex and there are no measurements of proton gradients, a more thorough justification of this statement is required.

(12) 2. The authors conclude that DNP is actively transported into the mitochondria instead of Pi at DNP concentrations below uncoupling phenomena. Competition is not measured explicitly, but is inferred indirectly through concentration-dependent inhibitor effects. A thorough discussion of the rationale leading to this conclusion is necessary.

(13) 3. The authors need to identify which of their results are not found by Hanstein and Hatefi. Why are the conditions used by Hanstein and Hatefi, "unfavorable" ?

(14) 4. The models of figure 3 are not adequately discussed in the manuscript. If the are of no value, eliminate them.

(15) 5. Present the data for Cd^{2+} inhibition if it is relevant or delete this section.

(16) 6. The data reported in the Results must be reconciled with the data shown in fig 1.
1. p.5 l. 22 The data of fig 1 do not indicate that 20 nmole NSPM / mg mito. was utilized.
2. p.6 l 1-4 The data of Panel A do not quantitatively support the authors statement s.

(17) 7. Provide the $K_{0.5}$' s for the detergent effects on oxid. phos., the State 4 to State 3 transition and the solubilization of mitochondrial protein.

EJB MS No. 95.0811P Report No.: 1

Referee: please complete if appropriate

- ☐ Table(s) ....can be eliminated
- ☐ Table(s) ....can be shortened
- ☐ Fig(s) ....can be eliminated
- ☐ Fig(s) ....can be shortened

→ exogenous ↓

This work is essentially based on the measurement of the endogenous glutathione level. However, no information about the method of glutathione determination is given. So it cannot be judged how these results have been obtained. It is very surprising that the author finds only very low concentrations of GSH, much lower than usually reported in the literature. Therefore, it is doubtful that these mitochondria are well coupled. Figure 1 is not understandable even after carefully reading the text. Also, Table 1 is quite confusing since there are no explanations in the legend.

not correct / not correct! / is given / well coupled / expl.

All the conclusions are highly speculative. For example, on page 14: "Respiratory control ratio is dependent upon glutathione bound to 30 kDa membrane proteins". is explained

EJB MS No. 95.0811P Report No.: 2

This manuscript, 2nd in a series of 4, by Kiehl Reinhold presents data interpreted as evidence that glutathione is an endogenous regulatory factor for the mitochondrial phosphate/proton symporter. The primary evidence for this seems to be that the quantity of membrane associated, oxidized glutathione (GS-SG) is decreased with respiration in the presence of Pi, that Cd^{2+} inhibits ^{33}Pi accumulation in mitochondria and that there is competition between the DNP analog, 2-azido-4-nitrophenol, and a sulfyhydry reagent, N-phenyl-N'- n-nonyl-thiourea (NPTU).

This manuscript, with the addition of the GS-SG measurement and the substitution of NPTU for NSPM in the DNP analog study provides data similar to that obtained in the first manuscript. As in the 1st manuscript the central issues are the paucity of experimental data including the criteria for the specificity of Cd^{2+} and NPTU for the various mitochondrial transporters and whether, in the absence of controls, the binding of a 2,4-dinitrophenol analog, 2-azido-4-nitrophenol is arguably to the Pi / H symporter. → explain 0,5 nmol/g → 0.05 ~ 1/10 2 diff.

In addition, the manuscript needs to be edited to remove techniques provided in the Methods section which are not utilized in the Results and to refocus the rambling discussion (which seems editorial in nature) to a discussion of the data derived from the investigation. In view of their similar focus, I would also suggest that any revision include combining these first two manuscripts. → 1+2 ?

Because of the extensive nature of this manuscripts inadequacies, I would reject these manuscripts rather that seek a revision.

EJB MS No. 95.0812P Report No.: 1

Referee: please complete if appropriate

☐ Table(s) .....can be eliminated
☐ Table(s) .....can be shortened
☐ Fig(s) .....can be eliminated
☐ Fig(s) .....can be shortened

English

This paper is a pure theoretical speculation without any experimental results. There is no real evidence for the wild speculation of energy transfer in the mitochondria and the role of the ATPaseis or ATP synthesis in a K^+ transport.

Not only the content but also the concept of the paper does not permit publication.

EJB MS No. 95.0812P Report No.: 2

This 3rd manuscript by Kiehl Reinhold presents an hypothesis that :
(1) mitochondrial ATP synthesis is due to a M_r : 30 kDa protein functioning as the Pi / H^+ symporter. The role of oxidized glutathione is to serve as a catalyst in a mechanism utilizing phosphoryl transfer via activated disulfides and sulfenyl phosphate.
(2) The mitochondrial F_0F_1-ATPase is a K^+ - pump.

For publication in the European Journal of Biochemistry I would expect an hypothesis to be based on strong experimental evidence. This appears lacking because most of the authors investigations are presented either as abstracts, his original dissertation or within this series of manuscripts. I would not discourage original or creative thought, but convincing experimental evidence must be presented for each tenant including:

1. A M_r : 30 kDa functions as a Pi / H^+ transporter.
2. This protein is the exclusive site of labeling by 2-azido-4-nitrophenol.
3. Sulfhydryl group reagents specifically inhibit this function and this protein.
4. Oxidized glutathione is essential for ATP synthesis by the M_r : 30 kDa protein.
5. K^+ transport by the mitochondrial F_0F_1-ATPase.

Again the authors style is confusing and it is possible that with a thorough revision his arguments and experimental evidence may appear more credible. However, until the body of experimental evidence is acceptable, I would recommend that this manuscript, which is largely dependent upon that data, be rejected.

English

European Journal of Biochemistry

Zürich, 23/05/95

Dr. Reinhold Kiehl
Laboratory and Research for
Mol. Medicine/Biology
Amselweg 12
D-93437 FURTH IM WALD
Germany

Reference no.: 95-0813

Transport and ATP synthesis in mitochondria
IV. K+-transport: Evidence for mitochondrial F0F1-ATPase being a K+-pump

by

Kiehl Reinhold,

Editor: Pettersson

Dear Dr. Kiehl,

Thank you for submitting your manuscript.

Manuscripts cannot be published in the Journal without a specific statement from the authors that the work is not being, and has not been, published elsewhere, and that all authors approve its submission.

Please sign the enclosed copyright form and the computer questionnaire and return them by regular mail to the Editorial Office.

You will be advised of the Editor's decision in due course.

Yours sincerely,

Dr. Lewis Rowett
Editorial Manager

Encl.: Copyright form
Computer questionnaire

Editorial Office: Apollostrasse 2, Postfach
CH-8032 Zurich, Switzerland

NEW TELEPHONE & FAX NUMBERS:
TEL. +41 (1) 388 20 40
FAX +41 (1) 388 20 41

European Journal of Biochemistry

Zürich, 25/07/95
23/05/95

I - IV.

Dr. Reinhold Kiehl
Laboratory and Research for
Mol. Medicine/Biology
Amselweg 12
D-93437 FURTH IM WALD
Germany

Reference no.: 95-0813

Transport and ATP synthesis in mitochondria
IV. K+-transport: Evidence for mitochondrial F0F1-ATPase being a K+-pump

by

Kiehl Reinhold

Editor: Pettersson

Dear Dr. Kiehl,

Thank you for submitting your manuscript.

I regret to inform you that the Editor responsible for the manuscript has advised me that it cannot be accepted for publication in the Journal. The referees' reports are enclosed for your information.

The top copy of your manuscript will be returned to you by separate printed-matter mail.

Yours sincerely,

L. H. Rowett

pp. Philipp Christen

Chairman of the
Editorial Board

Encl.: 2 reports
Copy : Editor

Editorial Office: Apollostrasse 2, Postfach
CH-8032 Zürich, Switzerland
Tel.:
Fax:

NEW TELEPHONE & FAX NUMBERS:
TEL. +41 (1) 388 20 40
FAX +41 (1) 388 20 41

EJB MS No. 95.0813P Report No.: 1

Referee: please complete if appropriate

- ☐ Table(s) .....can be eliminated
- ☐ Table(s) .....can be shortened
- ☐ Fig(s) .....can be eliminated
- ☐ Fig(s) .....can be shortened

English

The author presents a large number of difficult to understand experiments to support his idea that the mitochondrial ATP synthase is actually a K^+ pump. One of the main errors of the author is to assume that there is a high physiological K^+ gradient between the cytosol with about 175 mM and the matrix of the mitochondria with about 1 mM K^+. This is in contrast to all what is known about mitochondria that there is only a minor K^+ gradient. It might be that the mitochondria are damaged have lost most of their endogenous K^+.

no well coupled

+ literature

The whole concept of proving that the ATP synthase is a K^+ pump is based on a number of experiments with fluorescent dyes used as indicators of membrane potential and of pH. The author uses a mixture of different hydrophilic or hydrophobic SH-reagents which give a wide variety of phenomenological results which cannot be lined up in a logical proof of the function of the ATP synthase as a K^+ pump.

2

EJB MS No. 95.0813P Report No.: 2

English

This fourth manuscript by Kiehl Reinhold presents data interpreted as evidence of a K^+/ H^+-antiporter and an F_oF_1-ATPase-dependent K^+ transporter within the mitochondria and submitochondrial particles.

This manuscript presents a complex body of data describing the effects of the thiol reagent NSPM on mitochondrial functions within intact mitochondria and submitochondrial particles including respiration, swelling ΔpH, $\Delta \varphi$, catalytic partial reactions, NADH oxidation and the release of mitochondrial K^+. These data provide evidence of K^+ release from the mitochondria following reaction with NSPM as well as mitochondrial swelling and respiratory uncoupling as well as a variety of interrelated activities but seem to me to provide little evidence for a specific K/H antiporter.

?

As in the previous manuscripts, I have difficulty understanding what the author is saying and this alone requires a significant revision to clarify his intent. Specific points such as the central evidence for a K/H antiporter or a F_oF_1-ATPase-dependent K^+ transporter become obscured by a plethora of experimental variables whose relationship to the central thesis is poorly articulated. I could not even understand the object of the complex series of experiments reported in figures 5-11 utilizing the submitochondrial particles.

← engl.

It is not necessary to explain selfexplan. results, data by descriptio

There are similar concerns about data mentioned by the author to establish the identity of the ^{14}C-NSPM and ^{3}H-picrylacetate -labeled proteins where referenced experiments are reported exclusively in abstracts or in an unsubstantiated listing within the Discussion of the second manuscript of this series.

in this paper to

→ and P.4 !

→ weight !!

This manuscript seems to be a global essay of mitochondrial function and does not provide convincing evidence of the identity of the K^+ transporters. My suggestion would be for the author to eliminate data that is not directly relevant and have someone more familiar with English revise the manuscript. I recommend rejection of the present manuscript, because I am not assured that the author can accomplish this task.

←

EJB MS No. 95.0813P

Referee: please complete if appropriate

- ☐ Table(s) .....can be eliminated
- ☐ Table(s) .....can be shortened
- ☐ Fig(s) .....can be eliminated
- ☐ Fig(s) .....can be shortened

English

The author presents a large number of difficult to understand experiments to support his idea that the mitochondrial ATP synthase is actually a K^+ pump. One of the main errors of the author is to assume that there is a high physiological K^+ gradient between the cytosol with about 175 mM and the matrix of the mitochondria with about 1 mM K^+. This is in contrast to all what is known about mitochondria that there is only a minor K^+ gradient. It might be that the mitochondria are damaged have lost most of their endogenous K^+.

no well coupled

yes

The whole concept of proving that the ATP synthase is a K^+ pump is based on a number of experiments with fluorescent dyes used as indicators of membrane potential and of pH. The author uses a mixture of different hydrophilic or hydrophobic SH-reagents which give a wide variety of phenomenological results which cannot be lined up in a logical proof of the function of the ATP synthase as a K^+ pump.

+ literature

2 Vol

EJB MS No. 95.0813P Report No.: 2

English

This fourth manuscript by Kiehl Reinhold presents data interpreted as evidence of a K^+/ H^+-antiporter and an F_oF_1-ATPase-dependent K^+ transporter within the mitochondria and submitochondrial particles.

This manuscript presents a complex body of data describing the effects of the thiol reagent NSPM on mitochondrial functions within intact mitochondria and submitochondrial particles including respiration, swelling ΔpH, $\Delta \varphi$, catalytic partial reactions, NADH oxidation and the release of mitochondrial K^+. These data provide evidence of K^+ release from the mitochondria following reaction with NSPM as well as mitochondrial swelling and respiratory uncoupling as well as a variety of interrelated activities but seem to me to provide little evidence for a specific K/H antiporter.

2

As in the previous manuscripts, I have difficulty understanding what the author is saying and this alone requires a significant revision to clarify his intent. Specific points such as the central evidence for a K/H antiporter or a F_oF_1-ATPase-dependent K^+ transporter become obscured by a plethora of experimental variables whose relationship to the central thesis is poorly articulated. I could not even understand the object of the complex series of experiments reported in figures 5-11 utilizing the submitochondrial particles.

← engl.

It is not necessary to explain all explain results, data by description

There are similar concerns about data mentioned by the author to establish the identity of the ^{14}C-NSPM and ^{3}H-picrylacetate -labeled proteins where referenced experiments are reported exclusively in abstracts or in an unsubstantiated listing within the Discussion of the second manuscript of this series.

in this paper to

→ and P.4 !

→ right !!

This manuscript seems to be a global essay of mitochondrial function and does not provide convincing evidence of the identity of the K^+ transporters. My suggestion would be for the author to eliminate data that is not directly relevant and have someone more familiar with English revise the manuscript. I recommend rejection of the present manuscript, because I am not assured that the author can accomplish this task.