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Commentary by

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on 'The oxidation of glutamate by rat-heart sarcosomes'
P. Borst and E.C. Slater
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When the research leading to the publication of this Preliminary Note was started, we were not interested in glutamate oxidation per se. It was just one of the substrates used in studying the mechanism of oxidative phosphorylation.

In 1953, I had proposed that the energy from the redox reactions of the respiratory chain is primarily stored in a non-phosphorylated high-energy intermediate and that this intermediate could either react with P_i and ADP to form ATP or could be utilized directly for certain energy-requiring processes [1]. I further proposed that the energy of this intermediate is dissipated (by hydrolysis) by uncouplers, such as 2,4-dinitrophenol.

The evidence for the non-essentiality of phosphate for energy conservation, that was a basic tenet of this hypothesis, came from two lines of evidence. In the first place, I knew from my own earlier experiments and those of Walter Bonner [2] that, in contrast to glycolysis, phosphate is not necessary for the oxidation of NADH or succinate by the preparation of submitochondrial particles known as the Keilin and Hartree

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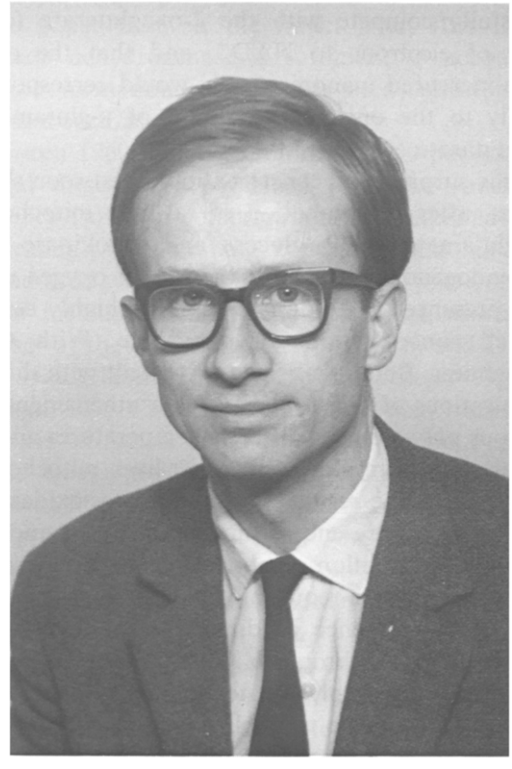

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heart-muscle preparation. Secondly, Loomis and Lipmann [3] had shown that 2,4-dinitrophenol can replace the inorganic phosphate necessary for maximal oxidation of glutamate by a crude preparation of kidney mitochondria known as cyclophorase, and this observation had been repeated by others, who had, however, raised some difficulties in the interpretation of the experiments. Since the idea of a non-phosphorylated intermediate was such an essential part of my hypothesis, it occurred to me, while writing a review in the summer of 1959, that it would be desirable to repeat the earlier experiments with the purer and more tightly coupled mitochondrial preparations that had since become available. I asked a young graduate student, Piet Borst, who had recently isolated sufficiently well-coupled tumour mitochondria to show phosphate respiratory control with glutamate as substrate, to check this point with just one simple experiment – one series of manometer flasks, done in a half day.

Since glutamate, the substrate used by Lipmann, is oxidized rapidly by mitochondria and was believed to deliver its electrons to NAD^+ or $NADP^+$, so that all phosphorylation steps in the respiratory chain would be traversed on their further passage to oxygen, I told Borst to use this as substrate. Of course, I knew that the product of the oxidation of glutamate – 2-oxoglutarate – would be further oxidized and that, as Judah [4] had shown, dinitrophenol does not abolish the requirement of phosphate for reaction of succinyl-CoA, the product of the oxidation of 2-oxoglutarate, but I was confident that glutamate, in the high concentration used, would successfully compete with the 2-oxoglutarate for the supply of electrons to NAD^+, and that the oxygen uptake measured manometrically would correspond essentially to the one-step oxidation of L-glutamate to 2-oxoglutarate.

To my surprise and consternation, Borst soon showed me that, after preincubation of rat-liver mitochondria with glutamate, ADP, glucose and hexokinase to remove endogenous phosphate, the rate of oxygen uptake in the presence of 2,4-dinitrophenol is highly sensitive to small concentrations of phosphate. With typical thoroughness, Borst confirmed this result with different concentrations of dinitrophenol, with other uncouplers, at various pH values, at different temperatures and with rat-heart sarcosomes as well as rat-liver mitochondria. In contrast to the results with glutamate, oxidation of succinate in the presence of uncoupler was found to be unaffected by addition of phosphate. It was concluded that the phosphate-requiring step in the respiratory chain, in the presence of dinitrophenol, lies between substrate and cytochrome *b*. The fact that it was not possible to show a phosphate requirement with 3-hydroxybutyrate, malate or proline as substrate could be due to the slow rate of oxidation of these substrates, matched by hydrolysis of a non-phosphorylated intermediate.

In any case, since the results certainly threw doubt on the validity of all my pronouncements concerning this point in recent lectures and reviews, I felt that it had to be published quickly and on July 24, 1959, Borst and I sent a Letter to 'Nature' entitled *Observations on the requirement for inorganic phosphate of dinitrophenol-stimulated oxidations*, with a long accompanying letter explaining why we thought that it was important.

Shortly afterwards, I travelled to Australia for a symposium in Canberra and a family visit, and did not return to Amsterdam, via U.S.A., until the middle of September. My travels caused some delay in answering queries from the Editor of 'Nature', mainly about the title, and the paper did not appear in print until October 31, under the title *Need for inorganic phosphate in oxidations stimulated by dinitrophenol* [5].

However, we were not aware that it had already appeared when, early in November, Professor F. Lynen visited our laboratory. When we told him about these experiments, Lynen asked if we were sure that there was no substantial oxidation beyond 2-oxoglutarate when glutamate was used as substrate. Although we gave the reasons stated above for our belief that this was not the case, the question brought to the surface worries in Borst's sub-conscious. He decided that the quickest way to test this was to try the effect of malonate on the oxygen uptake, since any succinate formed would certainly be rapidly oxidized and this would be inhibited by malonate. He did the experiment on November 11 and found 70% inhibition. He immediately confronted me with the result of this experiment and shortly afterwards showed that the amount (μmoles) of 2-oxoglutarate accumulating during the oxidation of glutamate, instead of being of the same order as the oxygen uptake (μatoms), as we had assumed, was less than 1% of it.

It was now clear that I had been too hasty in unnecessarily, and in panic, as it now appeared, amending one of the tenets of my mechanism of oxidative phosphorylation and on November 14, still not knowing that our Letter had already appeared, I asked the Editors to withdraw it. When they informed me that this was no longer possible, I asked them to publish a second Letter with the same title as the first. In this letter [6], which appeared on February 20, 1960, we stated that glutamate is not a suitable substrate for studying oxidative phosphorylation and respiratory control associated with the oxidation of reduced nicotinamide-adenine nucleotides and that there was no need to modify our earlier view that dinitrophenol acts on a non-phosphorylated high-energy intermediate of oxidative phosphorylation and this was further documented in a detailed study that we published in 'Biochimica et Biophysica Acta' in 1961, in which it is shown that phosphate does not stimulate oxygen uptake in the presence of dinitrophenol with any of a number of

NAD(P)-linked substrates that were examined [7]. This was not an easy investigation, since it was necessary to take account of partial inhibition by dinitrophenol, in the presence and absence of phosphate, of the oxidation of many of these substrates. Moreover, the unexpectedly high endogenous respiration in well-coupled liver mitochondria made it difficult to study poorly oxidizable substrates, such as 3-hydroxybutyrate. These difficulties are apparent on re-reading this paper, but the conclusions have stood the test of time and I consider it as one of my more important papers on oxidative phosphorylation.

This was an embarassing episode for me and a blow (fortunately temporary) to Borst's confidence, but was possibly a salutory demonstration to him of the fallibility of his teacher.

However, although it was clear that the requirement for inorganic phosphate was due to the 2-oxoglutarate → succinate step, problems remained. In particular, we found it still hard to believe that 2-oxoglutarate produced by the oxidation of glutamate, even in the small concentrations present in the initial stages (and the rate of oxygen uptake was constant for 30 min), would be immediately completely oxidized in the Krebs cycle to carbon dioxide and water, which was what was suggested by the 70% inhibition of oxygen uptake by malonate. The first step towards a solution of this problem is reported in the paper – a Preliminary Note – reproduced above.

When Borst found that, in the corresponding experiment with heart mitochondria, malonate inhibited oxygen uptake by 96.6%, it was clear that only a small fraction of the glutamate is oxidized via glutamate dehydrogenase and that the bulk is oxidized by a cycle involving succinate dehydrogenase. The 2-oxoglutarate, the oxidation of which is responsible for the phosphate requirement for glutamate oxidation in the presence of dinitrophenol, is formed, not by glutamate dehydrogenase, but by transamination with endogenous oxaloacetate:.

$$\text{glutamate} + \text{oxaloacetate} \rightarrow \text{2-oxoglutarate} + \text{aspartate}$$
$$\text{2-oxoglutarate} + 3/2\ \text{oxygen} \rightarrow \text{oxaloacetate} + H_2O + CO_2$$
$$\textit{Sum}: \text{glutamate} + 3/2\ \text{oxygen} \xrightarrow{(\text{oxaloacetate})} \text{aspartate} + H_2O + CO_2$$

This became known in our laboratory as the Borst transamination cycle (not to be confused with the Borst malate-aspartate shuttle for the transfer of reducing equivalents from extramitochondrial NADH to intramitochondrial NAD^+ [8]).

I had to take quite a lot of flack from a colleague more interested in nitrogen metabolism than I was for apparently ignoring the fact that he and others had already demonstrated the presence of transaminase in rat-liver mitochondria. In fact, I did not think that this was relevant, since current teaching was (and still is) that the function of transaminases, in liver at least, is to transfer amino groups to 2-oxoglutarate and that the glutamate formed is oxidized with formation of ammonia and regeneration of the 2-oxoglutarate, that is the reverse of the initial reaction of the transamination cycle. It is true that I had overlooked a paper by Müller and Leuthardt [9] from 1950, showing that glutamate could be converted to aspartate by rat-liver mitochondria, but these authors did not study the relative importance of the dehydrogenase and transamination pathways. Indeed, they concluded that the oxaloacetate required for transamination is formed by oxidation of the 2-oxoglutarate formed by the oxidative deamination of glutamate.

The discovery by Borst that, even in liver mitochondria, glutamate is almost completely oxidized to aspartate with little liberation of ammonia was quite unexpected and put into question the validity of Braunstein's [10] trans-deamination pathway for the conversion of amino-acid nitrogen into urea. Moreover, it left unanswered the question why the very active glutamate dehydrogenase in liver mitochondria was apparently not operating. These questions were raised by Borst in his full paper [11] and opened up a whole new field of research in our laboratory led by Joseph Tager, culminating in a paper in 1983 [12], dedicated to Professor A.E. Braunstein on the occasion of his 80th birthday, in which it is shown that bicarbonate in physiological concentrations, by inhibiting succinate dehydrogenase, stimulates the glutamate dehydrogenase pathway and inhibits the transamination cycle. Braunstein's mechanism is completely confirmed by the conclusion in this paper that, in the intact liver, flux through glutamate dehydrogenase is sufficient to account for ammonia formation required for urea synthesis from substrates such as alanine.

Between 1960 and 1983, more than 50 papers and 7 doctoral theses were published from the Amsterdam laboratory (some in collaboration with other laboratories) dealing with glutamate oxidation, ammonia formation and urea synthesis, directly flowing from this work. Authors include, in addition to Piet Borst, who is both Professor at the University of Amsterdam and Director of the Netherlands Cancer Institute, Joseph Tager, Ed De Haan, Rob Charles, all of whom also became full Professors in Amsterdam, as well as Sergio Papa and Ernesto Quagliariello of the University of Bari, Lars Ernster in Stockholm, John Williamson, Kath LaNoue and Jan Hoek (originally from Amsterdam) in Philadelphia, and Fred Meijer as well as Joseph's more recent collaborators – Ron Wanders and Bert Groen in Amsterdam.

The mistake of 1959 has been quite profitable!

I should explain why the word 'sarcosome' instead of mitochondria appears in the title of this paper. I was persuaded by my colleague, Ken Cleland [13], to use this term, since it had been used by Retzius [14], before

the word mitochondrion was introduced, to describe granules lying between the myofibrils. The term sarcosome, meaning muscle granule, also fits in nicely with the widely used term sarcoplasm to describe the non-fibril and non-nuclei fraction of the muscle, which is still retained in the term 'sarcoplasmic reticulum'. Moreover, at the beginning of the fifties, Cleland and I found it necessary to establish that the sarcosomes are truly the equivalent of mitochondria in other tissues and to examine their relationship to the Keilin and Hartree heart-muscle preparation that had been used in many classical papers on the respiratory chain by the Keilin school. It seemed a little illogical, at the outset of the investigation, to preëmpt the result by naming the granules 'mitochondria'. We were not alone in continuing into the 1960's to describe the interstitial granules in muscle as sarcosomes – the abundant granules in insect thoracic muscle were also usually called sarcosomes.

However, most biochemists referred to the heart granules as mitochondria and this became universal after David Green's very important large-scale preparation of what he called 'beef-heart mitochondria' which formed the basis of the classical studies by his school on the enzymes of the respiratory chain and oxidative phosphorylation. We then went with the stream.

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I would like to thank Professor P. Borst, who consulted his old workbooks to check and, in places, correct my memory of events of nearly 30 years ago.

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The oxidation of glutamate by rat-heart sarcosomes

The oxidation of glutamate by isolated mitochondria is generally believed to proceed via glutamic dehydrogenase, followed by oxidation of the α-ketoglutarate by the Krebs cycle. In the course of recent experiments on the mechanism of oxidative phosphorylation[1,2] we have found that only a small fraction of the glutamate oxidized by rat-heart sarcosomes follows this pathway. Most is oxidized to aspartate, according to reactions (1) and (2), the latter being that part of the Krebs cycle lying between α-ketoglutarate and oxaloacetate.

$$\text{Glutamate} + \text{Oxaloacetate} \longrightarrow \alpha\text{-Ketoglutarate} + \text{Aspartate} \quad (1)$$

$$\alpha\text{-Ketoglutarate} + {}^3/_2\,O_2 \longrightarrow \text{Oxaloacetate} + H_2O + CO_2 \quad (2)$$

$$\textit{Sum}\quad \text{Glutamate} + {}^3/_2\,O_2 \xrightarrow{\text{(Oxaloacetate)}} \text{Aspartate} + CO_2 + H_2O \quad (3)$$

That a product of the oxidation of succinate was necessary for the oxidation of glutamate was shown by the fact that the oxidation was inhibited 96.6 % by 0.02 *M* malonate, and that this inhibition was largely relieved by the addition of fumarate or malate (Table I). Pyruvate was ineffective while oxaloacetate gave variable results, probably because added oxaloacetate is very rapidly decarboxylated by heart sarcosomes. The glutamic dehydrogenase activity of swollen or sonicated sarcosomes was found to be very low. This is in agreement with results of VON EULER *et al.*[4] with whole heart extracts and with the very slow rate of the KREBS–COHEN[5] dismutation of α-ketoglutarate (in the presence of NH_3) by rat-heart sarcosomes[6].

TABLE I

EFFECT OF MALONATE AND KREBS-CYCLE INTERMEDIATES ON GLUTAMATE OXIDATION BY RAT-HEART SARCOSOMES[3]

*Substrate(s) and inhibitor***	*Rate of O_2 uptake (glutamate alone = 100)**		
	Mean	*Range*	*No. of expts.*
Glutamate	100		12
Glutamate + malonate	3.4	1.5–4.4	12
Glutamate + malonate + fumarate	75	63–85	4
Fumarate + malonate	20	17–24	3
Glutamate + malonate + malate	81	70–89	4
Malate + malonate	21	18–26	3
Glutamate + malonate + oxaloacetate	45	28–61	5
Oxaloacetate + malonate	38	23–47	4
Glutamate + malonate + pyruvate	9	4–14	2
Pyruvate + malonate	5	1–10	2

* The O_2 uptake was measured in a medium similar to that given in the legend to Fig. 1 of ref. 1; 0.02 *M* inorganic phosphate was present in all experiments, and the final pH was 7.5.

** Final concentrations: glutamate, 5–20 m*M*; oxaloacetate, 20–50 m*M*; other substrates and malonate, 20 m*M*.

Paper chromatography[7] showed that during glutamate oxidation aspartate —and no other amino acid—was formed. The aspartate formation was completely blocked by malonate and restored by the addition of malate or oxaloacetate. Under anaerobic conditions only oxaloacetate addition gave rise to aspartate. Preliminary experiments with the aspartic decarboxylase of *Nocardia globerula* described by

CRAWFORD[8] confirm that aspartate is formed during glutamate oxidation, in amounts close to that predicted by reaction (3) (see Table II).

These observations show that oxidation to aspartate is the most important pathway for glutamate oxidation by isolated rat-heart sarcosomes. It seems likely that oxaloacetate is the acceptor of the amino group in reaction (1), and that the reaction is catalysed by glutamic–aspartic transaminase, present in rat-heart sarcosomes[6]. Since this enzyme is known to be present also in mitochondrial preparations of other tissues, it is to be expected that reaction (3) is not restricted to rat-heart sarcosomes. Indeed we have found that aspartate is formed during the oxidation of glutamate by rat-liver mitochondria and Ehrlich ascites-tumour cell mitochondria.

TABLE II

ASPARTATE FORMATION DURING GLUTAMATE OXIDATION BY RAT-HEART SARCOSOMES

Reaction time (min)	*O_2 uptake (μatoms)*	*Aspartate formation** (μmoles)*	*ΔO/ΔAsp*
4	3.39*	1.23	2.8
8	6.74	1.79	3.8
12	9.24	2.47	3.7
16	12.37	3.60	3.4
20	15.02	3.89	3.9

* Mean of interpolated values in other flasks.
** Measured by the method of CRAWFORD[8] using differential manometers (gas volume, 6–7 ml).

It is clear that the glutamate–aspartate conversion should be kept in mind when glutamate is used as the substrate in studying mitochondrial oxidations. Moreover, the pathway of glutamate oxidation described here may be responsible for the rapid oxidation of glutamate in tissues with a low level of glutamic dehydrogenase, as proposed for brain by STRECKER[9].

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