Synthesis of Adenosine Triphosphate in Isolated Nuclei and Intact Cells

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1. It has previously been demonstrated that nuclei isolated from normal and neoplastic lymphoid cells are capable of oxygen-dependent ATP synthesis. In this paper it is shown that also the corresponding intact cells can synthesize ATP under those conditions in which nuclei can synthesize ATP. 2. In nuclei isolated from liver, kidney, rhabdomyosarcoma and osteosarcoma, oxygen-dependent ATP synthesis could not be demonstrated. The cells isolated from these tissues or tumours could not synthesize ATP either. The alternatives that such nuclei lost their ability for oxidative phosphorylation during the isolation procedure or that the process does not occur in these nuclei were explored. 3. Janus Green B, a vital stain for mitochondria, was used as a differential inhibitor of mitochondrial and nuclear ATP synthesis in intact cells. 4. Oxidative phosphorylation in mitochondria isolated from cells that had been incubated with various concentrations of Janus Green B (1–10 μ M) was seriously uncoupled, whereas at these concentrations oxygen-dependent ATP synthesis in isolated nuclei and in isolated cells were only inhibited to a small extent. 5. The results suggest that oxygen-dependent ATP synthesis in isolated cells measured under 'nuclear' conditions and in the presence of Janus Green B and Ca^{2+} is mainly due to nuclear oxygen-dependent ATP synthesis. The stimulation of cellular ATP synthesis by glucose was completely inhibited by Janus Green B. 6. It is tentatively concluded that the stimulation of ATP synthesis in isolated cells by glucose, which is not found in isolated nuclei, represents mitochondrial ATP synthesis, and nuclear and mitochondrial ATP synthesis can then be studied differentially in the intact cell. The possibility is considered that oxygen-dependent nuclear ATP synthesis is not a general property of cell nuclei.

It has been established in several Laboratories that isolated nuclei can synthesize ATP (Osawa, Allfrey & Mirsky, 1957; Creasey & Stocken, 1959; Betel & Klouwen, 1963; Klouwen & Betel, 1963). Until now this oxygen-dependent ATP synthesis has only been demonstrated in nuclei isolated from normal and neoplastic lymphoid and myeloid cells (Klouwen, 1965). In nuclei isolated from liver and kidney cells oxidative phosphorylation is absent although ATP synthesis by these isolated nuclei can be catalysed by adenylate kinase and probably by glycolysis (Klouwen, Betel, Appelman & Arts, 1965; Siebert, 1963). In contrast with thymus nuclei, these nuclei do not retain their nucleotides during isolation in 0.25 M-sucrose–3 mM-calcium chloride.

The absence of nuclear oxidative phosphorylation from liver, kidney and rhabdomyosarcoma may be because the process does not occur in these

nuclei or has been lost as a result of mechanical damage during isolation. A number of experiments were performed with thymus nuclei in which the effects of damaging treatments on ATP synthesis and ATP retention were studied and it was found that mechanical damage of nuclei can easily cause a loss of nucleotides and phosphorylating capacity. To avoid damage to nuclei during isolation, ATP synthesis was studied in intact cells under conditions used for synthesis of ATP in isolated nuclei. We now show that under special conditions, namely in the presence of Ca^{2+} and Janus Green B, which are inhibitors of mitochondrial oxidative phosphorylation, ATP synthesis determined in isolated cells represents mainly nuclear ATP synthesis.

Our experiments demonstrate that great variations exist in the cellular ATP content, ATP synthesis and the response of cellular ATP synthesis on the addition of glucose in different cell types. In the light of our knowledge of nuclear oxidative

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phosphorylation it is possible that both cytoplasmic and nuclear energy-generating sites are operative in various types of cells, the relative importance varying profoundly.

MATERIALS AND METHODS

Male albino rats weighing 130–150 g. and 6–8 weeks old were used. In a number of experiments erythropoiesis was stimulated in rat spleens by exposing rats at 0.33 atm. of air during three consecutive days, every day for 12 hr. During the nights the animals were kept under normal conditions and fed *ad libitum*. The spleens obtained after this treatment are referred to as 'stimulated spleens' and contained about 50% of erythropoietic cells of total cells present, whereas normal spleens contained only about 10%.

The mice used were CBA/Rij, C57BL/Rij and F₁ (CBA/Rij × C57BL/Rij) 7–10 weeks old, weighing 25–35 g.

Transplantable tumours. For CBA mice, tumour 19-A, for C57BL mice, tumour 2028, and for F₁ (CBA × C57BL) mice, tumour 30L, were obtained previously in this Laboratory from spontaneously occurring tumours. The myeloid leukaemia 121 was grown in RF mice. Further details about maintenance and the passage of tumours were described by De Vries & Vos (1958). Cell suspensions from thymus, spleen and lymph nodes were obtained by squeezing the minced tissue through nylon gauze. The cells were washed and centrifuged in an International Refrigerated Centrifuge PR II (head 269) for 5 min. at 300 g in 0.25 M-sucrose–3 mM-CaCl₂. Bone marrow was obtained from the femurs of rats. The cells were washed in a Tyrode solution (composition: NaCl, 135 mM; glucose, 5.5 mM; KCl, 2.7 mM; CaCl₂, 1.8 mM; MgCl₂, 0.5 mM; NaHCO₃, 8.4 mM; NaH₂PO₄, 0.4 mM; adjusted to pH 7.3 with CO₂). Rhabdomyosarcoma and osteosarcoma cells were obtained from tumours by a cell-dispersion technique with a medium containing trypsin and a special homogenizer (Reinhold, 1965). Kidney cells were isolated in a salt solution by using trypsin (Paul, 1961).

Liver cells were obtained after perfusion of the livers with 0.14 M-NaCl containing 0.5 mM-EDTA (Jacob & Bhargava, 1962). EDTA treatment is necessary to loosen the parenchymal structure. The minced liver was then squeezed through nylon gauze three times. The suspension was freed of broken cells and cell fragments by centrifugation for 10 min. at 300 g. With this technique completely stainable liver cells were obtained. The staining of cells and nuclei was performed according to the methods used for vital staining with 0.2% (w/v) eosine or 0.1% (w/v) trypan blue (Black & Berenbaun, 1964). The uptake of dye by cells has generally been regarded as an indication of non-viability or cell death.

T-1 cells and L-929 cells were cultured in an Eagle's suspension medium (Eagle, 1959). Glucose was not added to the medium.

All final cell suspensions were made in 0.25 M-sucrose–3 mM-CaCl₂ unless otherwise indicated.

Nuclei were isolated in 0.25 M-sucrose containing 3 mM-CaCl₂ according to the method described by Klouwen & Betel (1963). Liver and kidney nuclei were purified by layering over 0.34 M-sucrose (Hogeboom & Schneider, 1952). The cytoplasmic contamination of the nuclear preparations was judged microscopically after staining with methyl green–pyronin and by estimation of cytochrome c reductase (Rabinowitz & Bernard, 1957) and cytochrome oxidase (Smith, 1955). Dounce homogenizers with clearances on

diameter varying from 0.1 mm. to 0.04 mm. were used for final homogenizations.

ATP synthesis in isolated nuclei and cells was determined under the conditions described by Betel & Klouwen (1964). After isolation, the nuclei were shaken vigorously and divided in three equal parts. In the first part the nuclei were sedimented by centrifugation at 700 g for 7 min. and a HClO₄-soluble extract was prepared from the pellet with 1 ml. of 5% HClO₄/ml. of packed nuclei. This acid-soluble extract represents 'fresh' nuclei. To the acidified suspension an additional 1 ml. of HClO₄ was added. The two other parts were flushed with nitrogen and kept anaerobically for 15 min. at 30°. From one of these parts the nuclear pellet was obtained by centrifugation and after rapid decantation of the supernatant a HClO₄-soluble extract was prepared from the pellet. The other part was incubated aerobically for 30 min. at room temperature. The difference in ATP content in the nuclei kept under nitrogen and of the nuclei incubated aerobically after anaerobic incubation represents the amount of ATP synthesized.

ATP was determined in the neutralized acid-soluble extracts by an enzymic method. Phosphoglycerate kinase plus glyceraldehyde phosphate dehydrogenase catalyse the reaction:

The amount of NAD formed, which is equivalent to the amount of ATP present, was measured spectrophotometrically at 340 or 366 mμ (Adam, 1962).

DNA and RNA were determined according to the methods described by Klouwen & Betel (1963). Protein was determined according to the method of Gornall, Bardawill & David (1949).

Treatment of nuclei with ultrasonics was performed with a 9 kcyc./sec. 50 W Raytheon Sonic Oscillator (model S102A).

Isolation of mitochondria. Mitochondria were isolated in 0.25 M-sucrose containing 0.01 M-EDTA from intact lymphoma cells or thymocytes by using tight-fitting Dounce homogenizers. The homogenate was centrifuged for 7 min. at 900 g and the sediment consisting of cells and nuclei was resuspended and homogenized again. The supernatant obtained after centrifugation for 7 min. at 900 g was combined with the first supernatant and mitochondria were isolated by centrifugation for 10 min. at 13000 g. The mitochondrial sediment was suspended in 1 ml. of 0.25 M-sucrose–0.01 M-EDTA.

Oxidative phosphorylation was assayed for a 20 min. period at 30° in a medium consisting of: 0.15 ml. of potassium phosphate buffer, pH 7.35 (0.3 M); 0.1 ml. of glucose (1 M); 0.02 ml. of hexokinase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) (2 mg./ml.); 0.3 ml. of AMP (0.01 M); 0.3 ml. of sodium succinate (0.1 M); 0.2 ml. of MgCl₂ (0.1 M); 0.1 ml. of cytochrome c (type III from horse heart; Sigma Chemical Co., St Louis, Mo., U.S.A.) (0.8 mM); 0.05 ml. of NaF (0.33 M). Glucose 6-phosphate was determined enzymically (Hohorst, 1962).

RESULTS

ATP synthesis in cells and isolated nuclei. ATP synthesis was determined with nuclei isolated from different tissues.

In these experiments the amount of ATP synthesized after incubation at room temperature (18–22°) is not dependent of the actual temperature within this range because ATP synthesis has reached its maximum within 20 min. after the start of the incubation. At that time all endogenous nuclear AMP and ADP have been converted into ATP. The amount of ATP remains constant for at least 45 min. after the start of the aerobic incubation. Subsequently ATP contents of nuclei after aerobic incubation are never higher than the ATP contents of freshly prepared nuclei. Therefore ATP synthesis in the Tables is expressed as amounts (μ moles/mg. of DNA phosphorus) instead of rates (μ moles/mg. of DNA phosphorus/hr.).

In Fig. 1 the changes in ATP contents of isolated nuclei as a result of anaerobic treatment and subsequent aerobic incubation are shown. The results of a number of experiments are summarized in Table 1.

The nuclei isolated from spleens or 'stimulated spleens' (see the Materials and Methods section) showed very low ATP synthesis. The 'stimulated spleens' had high mitotic activity due to the proliferation of erythropoietic cells. A correlation between nuclear ATP synthesis and mitotic index did not exist. The nuclei that demonstrated ATP synthesis invariably showed a low percentage of staining. The cytoplasmic contamination of the thymus and lymphoma nuclei has been evaluated before (Klouwen & Betel, 1963; Klouwen, 1965). From the activities of cytochrome *c* reductase and cytochrome oxidase present in the nuclei it can be calculated according to Roodyn (1959) that this would account for 1–3% of mitochondrial protein or 5–10% of intact cells in the nuclear preparations. Free mitochondria are uncoupled in the incubation medium, which contains 3 mM-calcium chloride and hence cannot contribute to ATP synthesis. From Tables 1, 2 and 7 it can be seen by comparison of ATP content with synthesis of cells and nuclei that a contamination of 5–10% intact cells in nuclear

preparations cannot be held responsible for the ATP synthesis or ATP content in thymus- and F₁-lymphoma nuclei.

In subsequent experiments ATP synthesis was studied in intact cells under exactly the same conditions as were employed for nuclei.

The results of a number of experiments are summarized in Table 2. The ATP content of tissue-culture cells grown in suspensions and in ascites cells

Table 1. *ATP content and synthesis in nuclei isolated from normal and neoplastic tissues*

All incubations were performed at room temperature in 0.25 M-sucrose–3 mM-CaCl₂ at concentrations of about 5×10^8 nuclei/ml. of medium. See the Materials and Methods for technical details. The numbers of experiments are given in parentheses.

	ATP (μ moles/mg. of DNA P)		Stained nuclei (%)
	Content	Synthesis	
Thymus (rat)	0.55 (8)	0.30	20–35
Spleen (rat)	0.15 (3)	0.01	70–90
Stimulated spleen (rat)	0.19 (3)	0.02	70–90
CBA lymphoma (mouse)	1.20 (4)	0.60	5–25
C57BL lymphoma (mouse)	1.00 (2)	0.33	5–25
F ₁ lymphoma (mouse)	1.05 (6)	0.55	5–25
Myeloid leukaemia (mouse)	0.52 (2)	0.22	10–25
Rhabdomyosarcoma (rat)	0 (2)	0	90–100
Mesenterial lymph nodes (rat)	0 (3)	0	90–100
Liver (rat)	0 (3)	0	90–100
Kidney (rat)	0 (1)	0	90–100
Brain (rat)	0 (1)	0	90–100

Table 2. *ATP content and synthesis in intact cells*

Type of cell	ATP (μ moles/mg. of DNA P)		Stained cells (%)
	Content	Synthesis	
Thymocytes	0.88	0.52	5–15
Lymphocytes	0.73	0.25	10
Bone marrow	1.35	0.35	25
Ascites lymphoma L5178	2.42	1.86	10
Ascites lymphoma E.L. 4	4.32	1.80	10
L-929	4.70	1.49	0–5
T-1	2.13	1.53	5–10
Rhabdomyosarcoma	1.20	0	15–25
Osteosarcoma	0.80	0	10–15
Kidney	0.49	0	5
Liver	0	0	100

Fig. 1. Changes in ATP contents (μ mole/mg. of DNA P) of isolated thymus nuclei during anaerobic treatment and subsequent aerobic incubation.

Table 3. *ATP synthesis in thymocytes and nuclei*

The results are presented as means $\pm$ s.e.m. of four experiments.

	ATP (μ mole/mg. of DNA P)	
	Thymocytes	Thymus nuclei
Fresh	0.95 $\pm$ 0.05	0.50 $\pm$ 0.02
Anaerobic incubation	0.11 $\pm$ 0.03	0.09 $\pm$ 0.02
Aerobic incubation	0.69 $\pm$ 0.03	0.38 $\pm$ 0.01
Synthesis	0.58	0.29
Amount synthesized (% of initial amount)	61	57
Stained cells (%)	5-15	20-35

Table 4. *Effect of different homogenizers on ATP synthesis in isolated thymus nuclei*

The results are given as ranges for three experiments.

	Clearance of homogenizer (mm.) ...	ATP (μ mole/mg. of DNA P)		
		0.1	0.08	0.04
Content		0.50-0.70	0.50-0.70	0.30-0.50
Synthesis		0.24-0.34	0.24-0.34	0.14-0.20
Stained nuclei (%)		5-25	25-40	40-70

is considerably higher than the ATP content of cells obtained from tissues. Rhabdomyosarcoma, osteosarcoma and kidney cells, which contain ATP, do not show net ATP synthesis under 'nuclear' conditions, in contrast with thymocytes, lymphocytes, bone-marrow, ascites-lymphoma and cultured L and T cells. In the cell-culture experiments the suspension medium contained 6% of calf serum. The concentration of glucose in calf serum was 40mg./100ml. Hence the glucose concentration at the start of the cell culture was 130 μ M and decreased during the first 24hr. of cell culture to 40 μ M and to 4 μ M during the subsequent 24hr. of cell culture. Whether the ATP synthesis measured in intact cells represents nuclear ATP synthesis cannot be concluded from these results.

To investigate the contribution of cytoplasmic ATP synthesis under the conditions used, ATP synthesis was studied in both intact thymocytes and thymus nuclei. The results are summarized in Table 3.

These results show that the amount of ATP and the ATP synthesis in the isolated nuclei is lower than than the corresponding amounts in the intact thymocytes. The percentage of stained nuclei is generally higher than the percentage of stained cells. The differences observed in ATP content and ATP

Table 5. *Effect of 9kcyc./sec. ultrasonic treatment on nuclear ATP synthesis and ATP contents of thymus nuclei*

Ultrasonic treatment (sec.)	ATP (μ mole/mg. of DNA P)		Stained nuclei (%)
	Content	Synthesis	
0	0.53	0.22	40
5	0.24	0.07	70
10	0.19	0.04	70
20	0.06	0.00	90

synthesis could therefore partly be related to damage of the nuclei during the isolation procedure.

Effect of mechanical damage and ultrasonic treatment on ATP synthesis in isolated nuclei. To investigate the effect of mechanical damage of nuclei on the ATP synthesis, thymus nuclei were isolated by using Dounce homogenizers with different clearances between pestle and tube. The results are summarized in Table 4. With more tightly fitting pestles, decreases of ATP content, ATP synthesis and increase of stainability occurred, without concomitant loss of high-molecular-weight compounds like DNA, RNA and protein. Values for the DNA, RNA and protein contents of isolated thymus nuclei have been published before (Klouwen & Betel, 1963). Mechanical damage of the nuclei was probably also the cause of absence of ATP synthesis from nuclei isolated from rat mesenteric lymph nodes, because intact mesenterial lymphocytes contained nucleotides and synthesized ATP in sucrose-calcium chloride (see Tables 1 and 2). Nuclei isolated from calf mesenteric lymph nodes showed a relatively small ATP synthesis, and Creasey & Stocken (1959) demonstrated excellent ATP synthesis in rat lymph-node nuclei.

The effects of 9kcyc./sec. ultrasonic treatment are shown in Table 5. A rapid decrease of ATP content and ATP synthesis and increase of stainability was observed. The results presented in Tables 4 and 5 demonstrate that the damaging treatments cause a loss of metabolic activity and increase of stainability. The differences in ATP content and ATP synthesis between nuclei and cells can therefore at least partly be regarded as results of mechanical damage during the isolation.

Inhibition of ATP synthesis in intact cells. The possibility that in intact cells, the cytoplasm and especially the mitochondria contribute to the cellular ATP synthesis was investigated with the aid of inhibitors. Because it has recently been demonstrated (Betel & Klouwen, 1966) that mixtures of carbon monoxide + oxygen (2:98) can cause a

light-reversible inhibition of ATP synthesis in thymus nuclei, in contrast with previous results (McEwen, Allfrey & Mirsky, 1964; Betel & Klouwen, 1964), carbon monoxide was not used as differential inhibitor for nuclear and mitochondrial ATP synthesis.

Janus Green B, a vital stain for mitochondria, does not inhibit nuclear ATP synthesis (Mirsky, Osawa & Allfrey, 1956). Therefore experiments were performed with Janus Green B and the effect of this dye on oxidative phosphorylation in nuclei and intact cells was investigated. The results are summarized in Tables 6 and 7.

Table 6 shows that ATP synthesis in thymocytes and isolated nuclei is inhibited by Janus Green B to the same extent at the various concentrations used. At these concentrations ATP synthesis by isolated mitochondria is completely inhibited even at $1.3 \mu\text{M}$.

Mitochondria were isolated from F₁-lymphoma cells that had been incubated for 20 min. in the presence of $13 \mu\text{M}$ - and $1.3 \mu\text{M}$ -Janus Green B.

These concentrations of Janus Green B partly inhibit ATP synthesis in intact cells and nuclei (Table 6). The isolated mitochondria synthesized respectively 6.1 and $5.3 \mu\text{moles}$ of ATP/mg. of mitochondrial nitrogen as compared with $24.3 \mu\text{moles}$ of ATP/mg. synthesized under the same experimental conditions by mitochondria isolated from untreated lymphoma cells. Thymus

mitochondria isolated from thymocytes incubated with $13 \mu\text{M}$ -Janus Green B synthesized $0.3 \mu\text{mole}$ of ATP/mg. of mitochondrial nitrogen in 20 min.; control mitochondria synthesized $3 \mu\text{moles}$ of ATP/mg. of mitochondrial nitrogen in 20 min.

The phosphorylation experiments were normally performed in the presence of 3 mM-calcium chloride. Mitochondria were isolated from lymphosarcoma cells which had been incubated in the presence of various calcium chloride concentrations (namely 6 mM, 3 mM and 0.2 mM). They synthesized respectively $16 \mu\text{moles}$, $10 \mu\text{moles}$ and $18 \mu\text{moles}$ of ATP/mg. of mitochondrial nitrogen compared with $24 \mu\text{moles}$ of ATP/mg. synthesized by mitochondria isolated from cells that had not been in contact with Ca^{2+} . Isolated lymphoma mitochondria are, in the presence of 3 mM-calcium chloride, completely uncoupled.

Although no complete uncoupling of mitochondria isolated from cells incubated in the presence of Janus Green B was observed, the activity of the mitochondria was severely inhibited by this compound. The oxygen-dependent ATP synthesis determined in intact cells in the presence of these inhibitors is therefore mainly nuclear ATP synthesis.

A further attempt was made to evaluate the relative contribution of nuclear and cytoplasmic ATP synthesis in intact cells by using glucose (Table 7). Glucose does not stimulate ATP synthesis in isolated nuclei. In intact cells, as with ascites-lymphoma cells and cultured L cells, a marked stimulation by glucose of ATP synthesis was observed. In lymphoma cells and thymocytes the stimulatory effect of glucose was much smaller but was found consistently. In all cells studied in the presence of 3 mM-calcium chloride the stimulation of ATP synthesis by glucose was completely abolished by Janus Green B.

So far, stimulation of ATP synthesis by glucose has been demonstrated only in cells that show

Table 6. *Effect of Janus Green B on ATP synthesis in thymocytes and thymus nuclei*

Concn. of Janus Green B (μM)	Inhibition (%)	
	Thymocytes	Thymus nuclei
13	49	45
3	30	23
1.3	15	23
0.3	0	0

Table 7. *ATP content and synthesis in cells and isolated nuclei*

All incubations were performed in 0.25 M-sucrose–3 mM- CaCl_2 at room temperature.

	ATP (μmoles /mg. of DNA P)						
	L cells	T-1 cells	Ascites lymphoma	F ₁ lymphoma	Thymo-cytes	F ₁ -lymphoma nuclei	Thymus nuclei
Fresh	3.80	2.13	2.46	1.36	0.82	1.05	0.55
Anaerobic incubation: no addition	0.37	0.15	0.25	0.17	0.17	0.20	0.10
Aerobic incubation:							
(a) no addition	1.32	1.68	1.89	0.91	0.59	0.75	0.40
(b) plus 33 mM-glucose	2.57	2.30	2.30	1.06	0.68	0.75	0.40
(c) plus $13 \mu\text{M}$ -Janus Green B	1.06	—	1.46	0.65	0.38	0.51	0.29
(d) plus 33 mM-glucose and $13 \mu\text{M}$ -Janus Green B	1.23	1.80	1.76	0.81	0.43	—	—

oxygen-dependent ATP synthesis. The glucose effect seems to be mediated through the mitochondria because the effect is inhibited completely by Janus Green B. No glucose effect has been observed in cells in which no oxygen-dependent ATP synthesis could be demonstrated (rhabdomyosarcoma, osteosarcoma and kidney cells). These cells are isolated by a technique involving treatment with trypsin and are viable both from the point of view of staining and ED_{50} values (i.e. number of cells that result in 50% tumour takes after inoculation) in tumour titration studies (Reinhold, 1965). Moreover they contain large amounts of ATP. The results summarized in Table 7 show that, in the presence of Janus Green B and Ca^{2+} , in thymocytes and F_1 -lymphoma cells the amounts of ATP synthesized are the same as are synthesized in the isolated nuclei. Therefore any cytoplasmic contribution to the ATP synthesis in intact cells will be relatively small compared with nuclear ATP synthesis under these conditions and this oxygen-dependent ATP synthesis shows that there is nuclear oxidative phosphorylation in these cells.

DISCUSSION

Nuclei isolated from various types of cells, mainly of lymphoid origin and from myeloid-leukaemic cells, show the capacity of oxygen-dependent ATP synthesis (Klouwen, 1965).

In intact cells ATP synthesis could be demonstrated by using 'nuclear' conditions. It was shown (Table 6) that Janus Green B inhibits ATP synthesis in cells and in isolated nuclei to the same extent. In view of the strong inhibition of oxidative phosphorylation of mitochondria isolated from cells that had been incubated in the presence of Janus Green B a considerable contribution of mitochondrial ATP synthesis in intact cells would have resulted in a relatively greater extent of inhibition of ATP synthesis in cells by Janus Green B. At $1\mu M$ -Janus Green B oxidative phosphorylation in isolated mitochondria is uncoupled completely.

Table 7 shows that the extent of ATP synthesis in intact lymphoma cells and thymocytes in the presence of Janus Green B does not differ very much from the extent of ATP synthesis in the corresponding isolated nuclei.

From these results it is concluded that ATP synthesis measured under 'nuclear' conditions in the presence of Janus Green B and Ca^{2+} in cells, including cultured L and T cells and ascites-lymphoma cells, represents mainly the ATP synthesis of the cell nucleus.

The stimulation of cellular ATP synthesis by glucose points to the possible existence of other ATP-synthesizing sites in cells, becoming active

after the addition of glucose. The absence of the stimulation of ATP synthesis by glucose in isolated nuclei, together with the abolition of the glucose effect in intact cells by Janus Green B, suggest that the location of the glucose effect is in the cytoplasm. The absence of a glucose effect on ATP content in rhabdomyosarcoma, osteosarcoma and kidney cells cannot be explained at present.

The possibility has to be considered that oxygen-dependent ATP synthesis is not a general property of the isolated cell nucleus. The absence of this ability of isolated liver nuclei was shown by Klouwen *et al.* (1965). Isolated liver nuclei can, however, synthesize ATP by adenylate-kinase action. Conover & Siebert (1965) investigated the cytochromes present in rat-liver nuclei by low-temperature spectrophotometry. No cytochrome *b* and cytochrome oxidase could be detected and they concluded that the occurrence of a respiratory chain in liver nuclei is very unlikely. The absence of cytochrome oxidase from isolated liver nuclei has also been reported by Penniall, Currie, McConnell & Bibb (1964).

The morphological data available for thymus and liver nuclei isolated under mild conditions in 0.25M-sucrose do not show damage of the nuclear membrane (Allfrey, Littau & Mirsky, 1964; Gurr, Finean & Hawthorne, 1963).

Although the absence of oxygen-dependent ATP synthesis from isolated liver nuclei seems proved, this does not necessarily reflect the situation in the intact cells and the property of nuclear oxidative phosphorylation could be lost during isolation owing to mechanical damage. The correlation between nuclear oxidative phosphorylation and a low percentage of stained nuclei is apparent (Tables 1-5). The nuclei isolated from mesenteric lymph nodes are completely stained, and do not show oxidative phosphorylation, whereas the intact cells show good phosphorylating capacity. Any damage done to the isolated thymus nuclei either mechanically or by ultrasonic treatment led to a rapid loss of oxidative phosphorylation while the percentage of stained nuclei increased correspondingly. It is uncertain therefore whether the absence of oxidative phosphorylation in isolated nuclei reflects the situation in the intact cells, although the results obtained with nuclei and the corresponding intact cells indicate that in some cells, e.g. liver, kidney, osteosarcoma and rhabdomyosarcoma cells, the nucleus does not possess the capacity of oxidative phosphorylation.

At present the precise mechanism and location of the oxygen-dependent nuclear ATP synthesis is incompletely known and understood. The process shows the essential characteristics of mitochondrial oxidative phosphorylation like uncoupling by dinitrophenol and inhibition by oligomycin (Betel &

Klouwen, 1964). Glycolysis, tricarboxylic acid-cycle activity and electron-transport system are present in isolated thymus nuclei (McEwen *et al.* 1964). The presence of cytochrome *b*, cytochrome *c* and cytochrome oxidase in thymus nuclei has now been demonstrated (Betel & Klouwen, 1966). The inhibition of oxygen-dependent ATP synthesis in thymus nuclei by antimycin, Amytal, rotenone, carbon monoxide and cyanide suggests great similarities between the mitochondrial and nuclear electron-transport systems.

Since many cells and nuclei do not show oxygen-dependent ATP synthesis under 'nuclear' conditions, in these cells nuclear oxidative phosphorylation may be a relative unimportant process or perhaps completely absent. The morphology of various cells has to be considered too. The nucleus of the liver cell comprises about 10% of the cell volume whereas the nucleus of lymphoid cells occupies 40–50% of the total cell volume. These cells contain few mitochondria. Since thymus nuclei contain about 60% of the ATP present in thymocytes (Table 3), in this cell ATP is approximately evenly distributed over nucleus and cytoplasm. Whether this is true for the lymphoid-tumour cells has not been sufficiently explored.

Cells that possess an abundant number of mitochondria would generate ATP preferentially at cytoplasmic sites; whereas in cells with a poorly developed cytoplasm and a relatively large nucleus a number of metabolic activities, e.g. oxidative phosphorylation, glycolysis and protein synthesis, are also located in the nucleus.

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