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The Site of Synthesis of Mitochondrial Proteins in Krebs II Ascites-Tumour Cells

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At 22° in Earle's medium, Krebs cells synthesize proteins. After a brief 'pulse' with [^{14}C]valine followed by a 'chase' of [^{12}C]valine the radioactivity appears first in microsomes and is transferred after 'chase' to the cell sap. Kinetics of labelling of the mitochondrial protein are different from that of either microsomal or cell-sap protein. When Krebs cells in buffer are mixed with ribonuclease in water the nuclease penetrates the cell membrane. The ribonuclease-treated cells are still viable but have lost most of their cytoplasmic ribosomes (electron micrograph). Such cells still synthesize mitochondrial protein at near normal rate but synthesis of microsomal protein is severely inhibited. The results indicate that some mitochondrial proteins are synthesized independently of the microsome–cell-sap system.

Although isolated rat-liver mitochondria readily incorporate radioactive amino acids into protein, cytochrome *c* and malate dehydrogenase isolated from these labelled mitochondria are not radioactive (Roodyn, Suttie & Work, 1962). Cytochrome *c* and malate dehydrogenase are nevertheless typical mitochondrial proteins and in particular cytochrome *c* is not found elsewhere in the cell. These results forced us to devise experiments to distinguish between two possible hypotheses: either (*a*) biogenesis of mitochondria required integrated protein synthesis at two independent sites (one located inside and one outside the mitochondria), or alternatively (*b*) the incorporation of amino acids into insoluble proteins in a cell-free suspension of mitochondria is an artifact of the system and unrelated to true synthesis. It seemed to us that experiments with cell-free systems, however refined, would always remain suspect until the existence of two independent sites of synthesis was demonstrated in intact cells, particularly since experienced investigators had raised the spectre of bacterial contamination as a possible source of the 'cell-free' synthesis (Decken, Low & Sandell, 1966). As a suitable intact cell, our choice was the Krebs II ascites-tumour cell, which can readily be grown in quantity and maintained in simple media (Martin, Malec, Sved & Work, 1961). This cell, suspended in a glucose–phosphate medium, at 37°, incorporates amino acids into protein at great speed, but if the temperature is lowered to 22° the rate of synthesis becomes low enough to permit effective 'pulse'-labelling experiments. As shown in the present paper, the results of these experiments are most readily understood if it is assumed that there are in fact two independent routes for synthesis of the soluble and the insoluble proteins of mitochondria.

Further confirmation of this hypothesis was supplied by another series of experiments in which cells were subjected to osmotic 'shock' in the presence of ribonuclease. Ribonuclease treatment did not kill the cells, but it lowered the rate of synthesis of microsomal proteins without having much effect on the rate of synthesis of mitochondrial proteins.

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MATERIALS AND METHODS

Cells. Krebs II mouse ascites-tumour cells were propagated, collected, washed and counted as described by Martin *et al.* (1961).

Buffers. The three buffers used were phosphate-buffered saline (Martin *et al.* 1961), Earle's buffered glucose (Martin *et al.* 1961) and phosphate saline. This last buffer contained NaCl (40g.), KCl (1g.), Na_2HPO_4 (5·75g.) and KH_2PO_4 (1g.) in 5l. of water.

Sucrose–EDTA–nicotinamide. This solution contained (final concn.) sucrose (0·3M), EDTA (0·01M) and nicotinamide (0·03M); the pH was adjusted to 7·2.

Radioactive valine. DL-[^{14}C]Valine was used throughout these investigations. It had a specific activity of approx. 5mc/m-mole (The Radiochemical Centre, Amersham, Bucks.).

Radioactive proteins. Proteins were precipitated with trichloroacetic acid and washed free of contaminants in the usual way (Simkin & Work, 1957). Samples were counted with a thin window Geiger–Müller counter (efficiency about 15%).

Fractionation of homogenates. Krebs cells were washed in

Ca^{2+}-free Mg^{2+}-free phosphate-buffered saline and suspended in 0·3M-sucrose (1×10^8 cells/ml.). After 10min. in sucrose the cells were spun down, resuspended in fresh sucrose and disintegrated in an Ultra-Turrax disintegrator as described by Freeman (1965). KCl was added to a final concn. of 0·1M and the nuclei were removed (700*g* for 10min.). Mitochondria were collected by centrifuging for 10min. at 8000*g* and microsomes by spinning at 105000*g* for 2hr. The nuclear and mitochondrial fractions were resuspended in sucrose–EDTA–nicotinamide and again pelleted to remove adherent cytoplasm.

RESULTS

A number of attempts were made to 'pulse'-label Krebs-cell proteins with [^{14}C]valine at 37°, but the rate of incorporation of the isotope was so great that no satisfactory results were obtained. However (Table 1), if cells were incubated at 22° and [^{14}C]-valine was added, labelling of subcellular proteins occurred (after a brief lag) at a linear rate for 40min. This was equally true for the proteins obtained from nuclei, mitochondria, microsomes and cell sap. It is clear therefore that there is no objection to decreasing the rate of synthesis by lowering the temperature.

Before carrying out 'pulse'-labelling experiments, however, we wished to be sure that any labelled protein isolated from the mitochondrial fraction owed its radioactivity entirely to mitochondrial protein and not to any small quantity of adherent but more highly radioactive microsomal protein. This was achieved by washing the mitochondria with 0·3M-sucrose containing 0·01M-EDTA. As shown in Table 2, EDTA treatment breaks down the microsomal fraction and releases from it, in a soluble form, labelled protein that may have been inadvertently carried over into the mitochondrial fraction. The EDTA wash thus reduces very considerably the chance of spurious results.

Kinetics of labelling of proteins from subcellular fractions. It is evident, from the results given in Table 1, that at 22° as at 37° (Littlefield, Keller, Gross & Zamecnik, 1955) the microsome fraction is much more rapidly labelled during the first 5min. than any other fraction. If non-radioactive carrier valine was added in excess to the cell suspension at 22° after 5min., the increase in specific activity of whole-cell protein ceased within a few minutes, but isolation of protein from individual cell subfractions showed very striking differences between different fractions. The specific activity of nuclear protein continued to rise slowly; that of mitochondrial protein increased somewhat over the first 5min. after addition of the 'chase' and then remained constant. The microsome fraction behaved in the classical fashion, showing maximum specific activity at the time of addition of the 'chase' and fell rapidly thereafter. The soluble fraction also behaved in the expected and classical fashion, showing a lag in the first 5min. followed by a steady increase in specific activity after addition of the 'chase'. These results are shown in Fig. 1.

In several similar experiments, the 'chase' was added at 10 or 20min. instead of 5min. As might be expected, there was again the classical picture of a rapid fall in the specific activity of the microsome fraction and a corresponding rise in the cell-sap fraction, but, once again, the mitochondrial fraction showed a slight increase or no change after addition of the 'chase' (Fig. 1).

Though these results indicate that the radioactive proteins found in the mitochondria and in the cell sap have different origins, they are no more than suggestive of the existence of an independent protein-synthesizing mechanism within mitochondria. Confirmatory evidence of this possibility was therefore sought. It was thought that some of the well-known inhibitors of ribosomal protein

Table 1. *Time-course of incorporation of* [^{14}C]*valine into the proteins of Krebs II ascites-tumour cells at* 22°

Washed cells were suspended in Earle's medium (see the Materials and Methods section) at a concentration of 2×10^7/ml. and to each 5ml. of cells was added 0·17μc of [^{14}C]valine. Cells were disrupted and fractionated as described in the Materials and Methods section.

Fraction	Sp. activity of protein (mμc/g.)			
Time of incubation (min.)	5	10	20	40
Whole homogenate	68	260	520	906
Nuclei	40	153	334	629
Mitochondria	84	260	520	1047
Microsomes	193	562	830	1258
Cell sap	22	123	406	850

Table 2. *Release of radioactive protein from Krebs-cell microsomes by treatment with EDTA*

Cells were labelled as described in Table 1 for 5min. at 22°, disrupted and fractionated as before and the microsomal fraction was divided into four portions; each portion was suspended in 3ml. of 0·3M-sucrose containing the indicated concentration of EDTA and the microsomes were pelleted at 105000*g*.

Concn. of EDTA (mM)	Sp. activity of protein (mμc/g.) Pellet	Supernatant
0	252	43
1	207	48
3·3	149	155
6·6	80	244

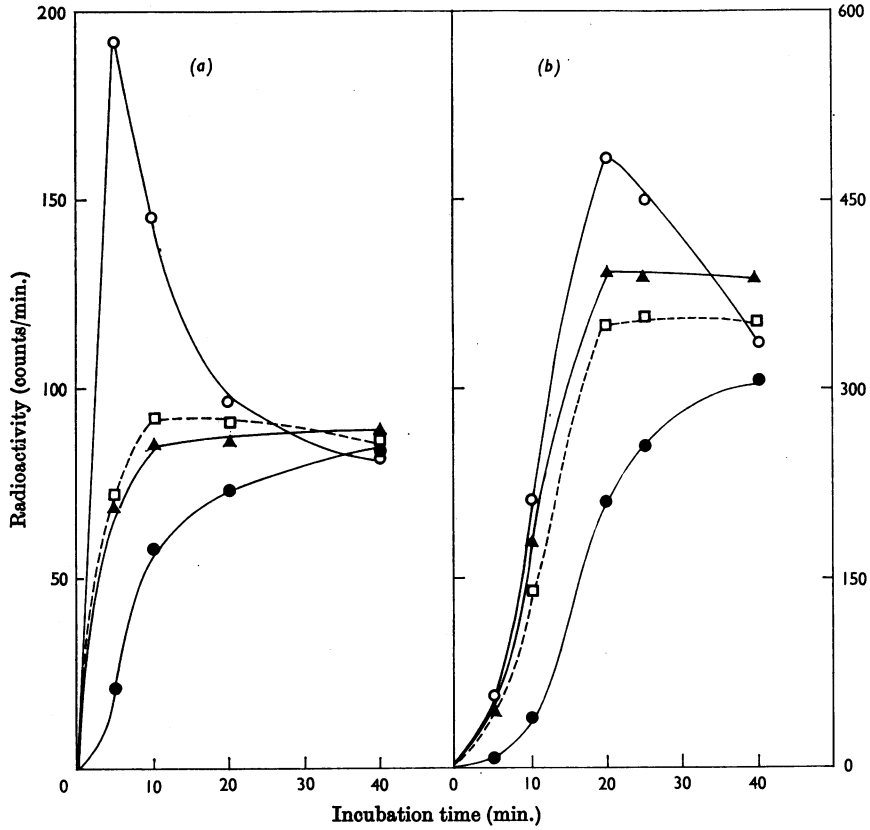


Fig. 1. Time-course of incorporation of [^{14}C]valine into the proteins of subcellular fractions of Krebs ascites-tumour cells before and after a 'chase' of non-radioactive valine. In (*a*) the 'chase' was added at 4 min. 40 sec., and in (*b*) at 20 min. The medium was Earle's medium. Incubations were at 22°. Cells were disrupted in the usual way and fractionated as described by Freeman (1965). Mitochondria were washed three times with sucrose–EDTA–nicotinamide (0·3 M, 0·01 M, 0·03 M) before adding trichloroacetic acid. For incorporation in the absence of 'chase', see Table 1. □, Whole homogenate; ▲, mitochondria; ○, microsomes; ●, cell sap.

synthesis such as puromycin or cycloheximide might selectively inhibit ribosomal protein synthesis without inhibiting at the same time the synthesis of mitochondrial protein. We failed, however, to find any low-molecular-weight inhibitor which would do this and we concluded therefore that, though there might be separate sites of protein synthesis within a cell, the chemical mechanisms of synthesis were likely to be closely similar throughout; any selective inhibitor would therefore have to distinguish between sites rather than between mechanisms. Very early in the study of biosynthesis in mitochondria, it was shown that ribonuclease did not inhibit incorporation of radioactive amino acids into the protein of isolated mitochondria (McLean, Cohn, Brandt & Simpson, 1958; Reis, Coote & Work, 1959), whereas it was well known that synthesis by the isolated ribosome–cell-sap system is susceptible to inhibition by traces of ribonuclease (Zamecnik & Keller, 1954). It seemed worth while therefore to seek conditions under which ribonuclease could be introduced into the cytoplasm of intact cells.

Effects of ribonuclease and osmotic 'shock'. Strange & Postgate (1964) reported that cold 'shock' or exposure to distilled water allowed penetration of ribonuclease into the cells of *Aerobacter aerogenes*. Despite the great difference in the cell type, similar methods were tried with Krebs cells. Three methods of 'shock' were tried; cold 'shock', transfer from hyperosmotic to iso-osmotic medium and transfer from iso-osmotic to hypo-osmotic and back to iso-osmotic medium. Exposure of cells to ribonuclease at the concentration of 100 μg./ml. at 22° had no effect on their rate of protein synthesis but sudden chilling from 22° to 0°

followed by treatment with ribonuclease caused slight inhibition of protein synthesis. The degree of inhibition obtained by this method was less than we sought and we therefore tried instead osmotic 'shock'. Transfer of cells from a hyperosmotic to an iso-osmotic medium caused some cell rupture and almost complete stoppage of protein synthesis, but transfer from an iso-osmotic to a hypo-osmotic medium and back to an iso-osmotic medium had a much less drastic effect and seemed to allow penetration of ribonuclease into the cells. The effect of increasing concentrations of ribonuclease in inhibiting protein synthesis in the osmotically 'shocked' Krebs cells is shown in Fig. 2. Concentrations of ribonuclease beyond 500 μg./ml. were not tried since the form of the inhibition curve indicated that 60% inhibition was likely to be near the maximum attainable under these conditions.

It is obvious that, in osmotic-'shock' experiments of the type described in Fig. 2, the number of possible variables is far too large for it to be practicable to cover all of them, but, to gain some idea of the effect of changes in the different parameters we varied, in turn, the time of exposure to osmotic 'shock', the concentration of ions during osmotic 'shock' and the temperature of the 'shock', since we thought these were likely to be the most important variables. It was found that, in sodium chloride, if the salt concentration during 'shock' was lowered below 0·02 M and held at this for 15 min. the cells were completely unable to incorporate radioactive valine when restored to iso-osmotic medium. The severity of osmotic 'shock' was greater at 37° than at 22°, but by far the most critical factor seemed to be the time of exposure to osmotic 'shock' and this was investigated in greater detail. The results of an experiment in which the time was varied between 1 and 40 min. are given in Table 3.

It was obvious from these results that osmotic 'shock' alone has a severe inhibiting effect on protein synthesis and it is noteworthy that the maximum selective inhibition due to ribonuclease seems to have been reached after 14 min. of osmotic 'shock'. In all subsequent experiments, periods of 15 min. or less were used.

Since the effect of osmotic 'shock' alone was so severe, one further variable was investigated. It seemed possible that the low incorporation values, produced by osmotic 'shock' alone, might be partly due to loss of or a dilution of available metabolizable substrates. To counteract this effect, we tried adding either glucose–sodium chloride or concentrated Earle's solution after osmotic 'shock'. This had a striking effect in restoring the rate of protein synthesis; thus, for example, cells 'shocked' for 15 min. at 22° and then restored to iso-osmotic

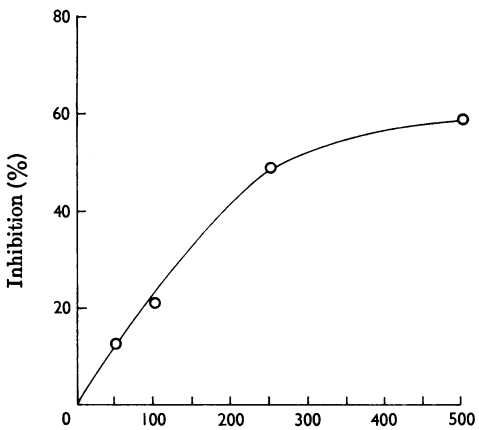


Fig. 2. Inhibition of incorporation of [^{14}C]valine into the protein of Krebs cells after osmotic 'shock' in the presence of ribonuclease. Each batch of $1·5 \times 10^8$ cells was suspended in 1 ml. of Ca^{2+}-free, Mg^{2+}-free phosphate saline (see the Materials and Methods section) and held at 22°. Then 7 vol. of water was added with the appropriate amount of ribonuclease and after 15 min. sufficient 2 M-NaCl was added to restore the osmoticity to normal. The temperature was raised to 37° and the cells were incubated for 15 min. with L-[^{14}C]valine (0·07 μc/ml.).

Table 3. *Effect of ribonuclease on protein synthesis by Krebs cells subjected to osmotic 'shock' for different times*

Washed Krebs cells (6×10^8) were suspended in 4 ml. of Ca^{2+}-free Mg^{2+}-free phosphate saline and poured into 28 ml. of water containing ribonuclease (100 μg./ml.). At various times thereafter samples were removed and the osmotic pressure in each sample was restored to iso-osmotic by addition of 2 M-NaCl. (Since Krebs cells can be maintained over long periods at 37° in Earle's medium it is assumed that Earle's medium is approximately iso-osmotic for this cell). The temperature was raised to 37° and DL-[1-^{14}C]valine was added (0·09 μc/ml.). Incorporation of radioactivity was allowed to proceed for 15 min.

Period of 'shock' (min.)	Ribonuclease (μg./ml.)	Protein (counts/min./10^8 cells)	Inhibition by ribonuclease (%)
1	0	3330	
1	100	2660	20
5	0	1053	
5	100	800	24
14	0	454	
14	100	290	36
25	0	244	
25	100	154	37
40	0	76	
40	100	72	5

Table 4. *Selective inhibition by ribonuclease of labelling of microsomal proteins of Krebs cells by* [^{14}C]*valine*

A suspension of Krebs cells ($1{\cdot}5 \times 10^8$/ml.) was poured into 7 vol. of a solution of ribonuclease in water (100 μg.ml.). After the indicated time at 22° the solution was made iso-osmotic by addition of 2 M-NaCl containing glucose (final concn. 0·01 M). Radioactive valine (0·1 μc/ml.) was added and the cells were incubated at 37° for 15 min., chilled to 0° and disrupted in the usual way. A portion of the homogenate was used to measure specific activity of whole cell protein and the remainder was fractionated as described in the Materials and Methods section. In control batches of cells, ribonuclease was omitted during osmotic 'shock'. In Expt. A, the cells were incubated for 15 min. at 37° in glucose–saline before adding valine. In Expts. B and C preincubation was for 10 and 5 min. respectively. Values in parentheses in Expt. C are counts for a similar experiment in which glucose was omitted.

Fraction		Duration of 'shock' (min.)	Radioactivity in protein (counts/min./mg.)		Change due to ribonuclease
			No ribonuclease	Ribonuclease	
Expt. A	Homogenate	5	115	84	−27
	Mitochondria	5	45	48	+6
	Microsomes	5	126	89	−30
Expt. B	Homogenate	10	69	36	−48
	Mitochondria	10	55	43	−22
	Microsomes	10	181	70	−61
Expt. C	Homogenate	15	178 (26·7)	56 (17·7)	−68
	Mitochondria	15	126 (25·4)	76 (22)	−39
	Microsomes	15	215 (37)	66 (23)	−70

sodium chloride incorporated [^{14}C]valine at less than one-third of the rate of cells resuspended in Earle's solution. The superiority of Earle's solution over sodium chloride was undoubtedly due to its content of glucose since glucose (0·01 M final concentration) in sodium chloride had a similar effect (see Table 4). Glucose–sodium chloride was preferred to Earle's medium since the latter contains Mg^{2+} and Ca^{2+} ions, which tend to inhibit ribonuclease and in the case of Ca^{2+} to inhibit also oxidative phosphorylation.

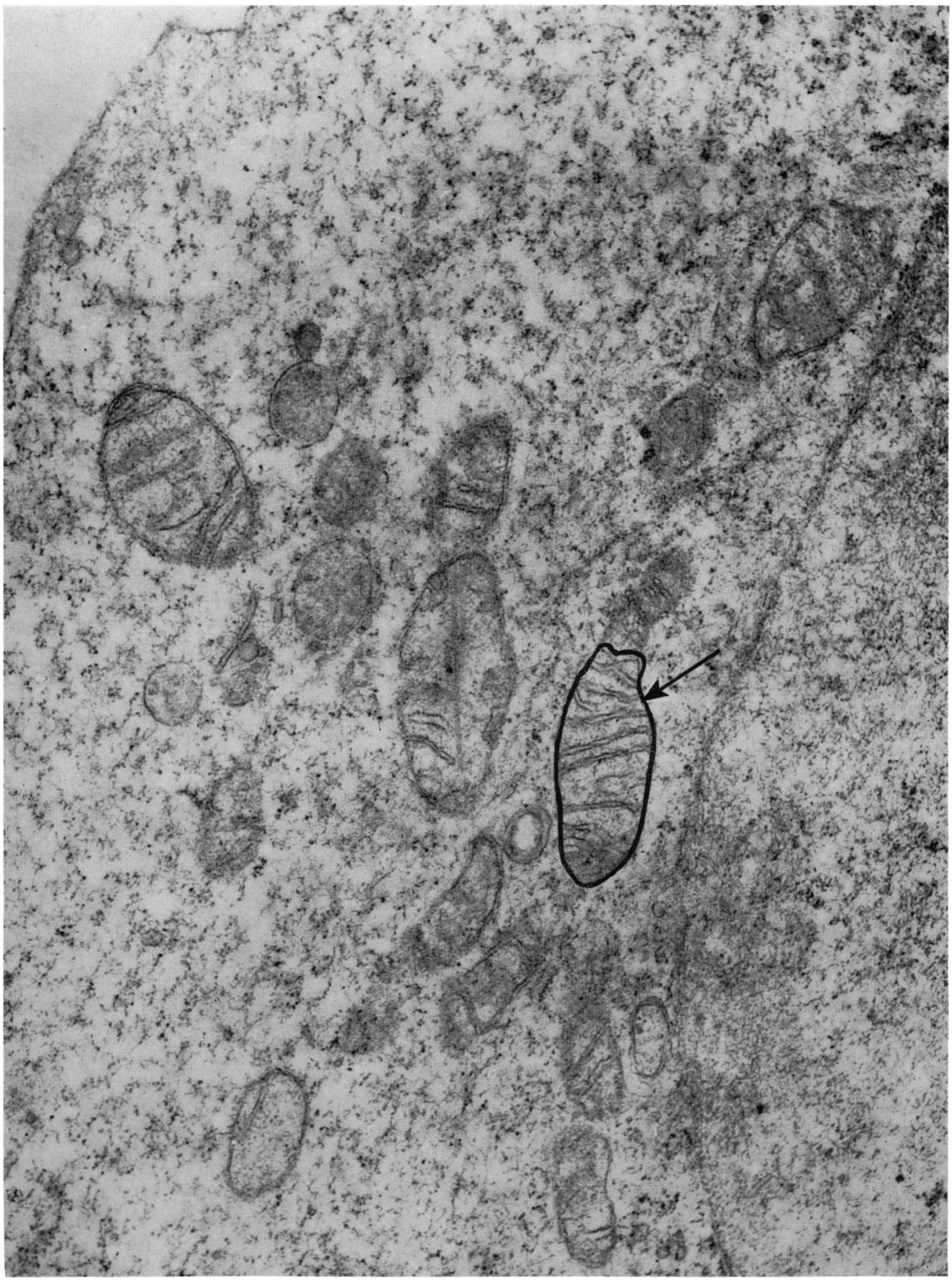
Selective inhibition of microsomal protein synthesis by ribonuclease. The results reported above indicated that ribonuclease does enter Krebs cells during the period of osmotic 'shock'. Since protein synthesis by isolated mitochondria is not inhibited by ribonuclease (Reis *et al.* 1959), it was decided to compare the percentage inhibition of labelling of mitochondrial and microsomal proteins of Krebs cells after osmotic 'shock' in the presence of ribonuclease. In three experiments successive batches of cells were suspended in phosphate-buffered saline and poured into water containing ribonuclease, and after periods of 5, 10 or 15 min. the osmoticity was restored to normal by addition of a glucose–sodium chloride mixture. The cells were then incubated with [^{14}C]valine for 15 min. before rupture. Mitochondria and microsomes were separated and the specific activity of the protein from each was determined. The results, given in Table 4, clearly show that, in whole cells, ribonuclease inhibited synthesis of microsomal proteins much more than it inhibited synthesis of mitochondrial proteins.

Viability of Krebs cells after osmotic 'shock' in the presence of ribonuclease. To check whether either osmotic 'shock' alone or osmotic 'shock' in the presence of ribonuclease had seriously disorganized the normal metabolism of Krebs cells, we compared the behaviour of normal cells with that of 'shocked' cells as propagation stock for the production of new tumours. As the results in Table 5 show, mice injected intraperitoneally with osmotic-'shock'-treated cells, or with osmotic-'shock'-plus-ribonuclease-treated cells, developed new tumours at the same rate and gave the same yield of tumour cells as did mice injected with an equal quantity of fresh cells.

In a second similar experiment, three groups of 24 mice were injected. In the first group all animals received 1×10^7 freshly washed Krebs cells, in the second group all animals received 1×10^7 'shocked' cells and the third group were injected with an equal

EXPLANATION OF PLATES 1–3

Electron micrographs of thin sections of Krebs cells. The sections were prepared by Dr J. A. Armstrong from cells fixed for 1–2 hr. in phosphate-buffered osmium tetroxide. The cells were embedded in Epikote 812 and sectioned in an LKB ultramicrotome. Sections were stained with uranyl acetate and lead nitrate and photographed with a Philips EM75 and a Philips EM200 electron microscope. Plate 1, normal cell; Plate 2, cell treated with 400 μg. of ribonuclease/ml. and osmotic 'shock'; Plate 3, cell osmotically 'shocked' in the absence of ribonuclease. Arrows indicate typical mitochondria. Magnification: × 32000.



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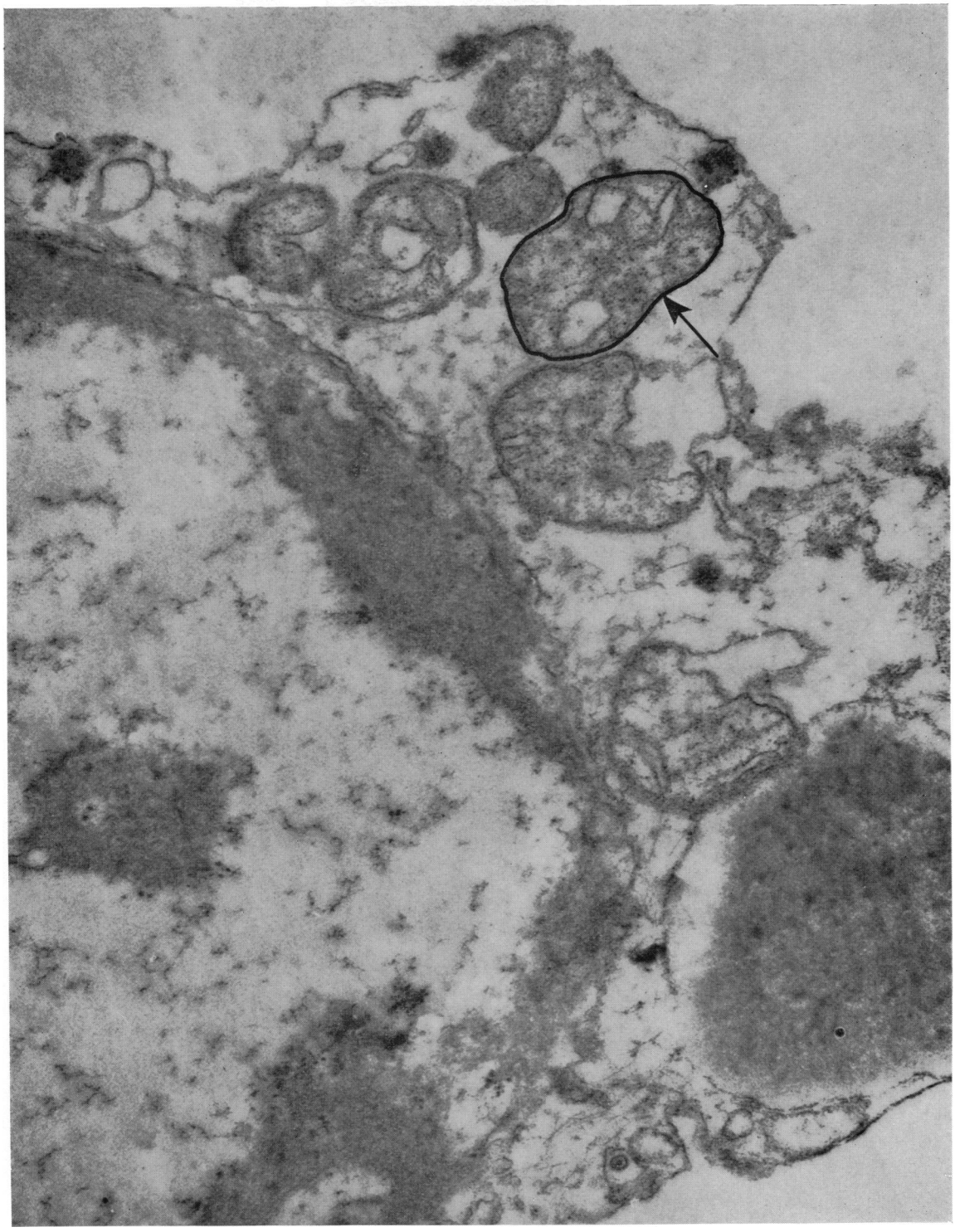

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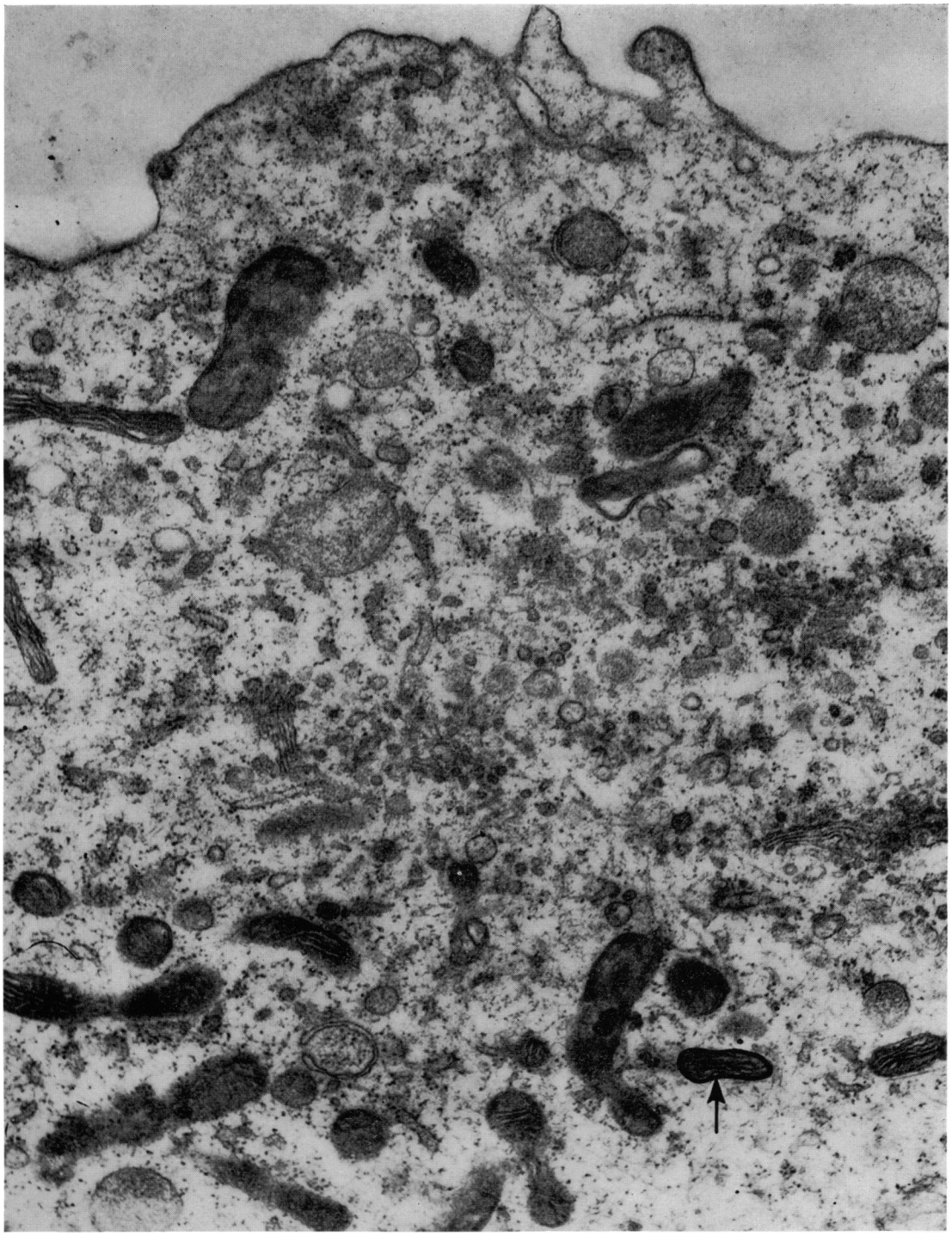

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Table 5. *Production of ascitic tumours by Krebs cells after osmotic 'shock' with or without ribonuclease*

Fresh Krebs cells were suspended in Ca^{2+}-free Mg^{2+}-free phosphate saline, divided into three portions and one portion of cells was used as control, one portion was shocked by pouring the suspension into aqueous ribonuclease (100 μg./ml.) and one portion was poured into water. After 10 min. 2 M-NaCl was added to restore osmoticity and the cells were centrifuged down and resuspended in phosphate-buffered saline. The cell concentration was adjusted to 1×10^8 cells/ml. and three groups of mice (15 in each) were injected with the three types of cell. Each mouse received 0·1 ml. of cell suspension. Ascitic fluid was collected 9 days after the injections.

Mouse no. in group	Yield of ascitic fluid (ml.) Normal cells	'Shocked' cells	'Shocked'+ ribonuclease
1	17·5	9·5	16·0
2	3·5	4·5	6·0
3	0·0	1·0	1·5
4	1·0	4·0	10·0
5	14·0	12·0	5·0
6	0·0	2·5	4·5
7	2·0	2·5	15·0
8	3·0	6·5	9·0
9	13·0	14·5	0·0
10	0·0	0·0	9·0
11	17·0	5·0	0·0
12	Dead	Dead	Dead
13	Dead	—	Dead
14	Dead	—	Dead
15	—	—	Dead
Total yield of fluid from 11 mice	71	62·5	76

quantity of cells 'shocked' in the presence of ribonuclease (as in Table 5). Each group of mice was weighed daily from day 2 to day 6, which is known to be the period during which normal Krebs cells show their maximum rate of growth (Kerr, 1963). The results are not shown in detail but there was no significant difference between the rate of growth of the normal cells, the 'shocked'-ribonuclease-treated cells and the cells 'shocked' in the absence of ribonuclease. The cell densities in the aspirated ascitic fluids were also indistinguishable.

Effect of ribonuclease and osmotic 'shock' on the morphology of Krebs cells. Since our biochemical investigations had indicated that microsomal protein synthesis was severely inhibited after exposure of Krebs cells to ribonuclease plus osmotic 'shock', it was decided to examine thin sections of such 'shocked' cells in the electron microscope. Our colleague, Dr J. A. Armstrong, very kindly carried out the sectioning, staining and electron microscopy. Sections of normal cells, cells exposed to

Table 6. *Effect of various antibiotics on the incorporation of radioactive amino acids into protein of isolated mitochondria*

The mitochondria were isolated in the usual way, washed three times in sucrose–EDTA–nicotinamide and suspended in medium B of Roodyn, Reis & Work (1961) supplemented with potassium succinate (0·01 M) and glucose (0·01 M). The suspension was divided into eight equal portions containing mitochondria at a concentration of about 3 mg./ml. and antibiotics as indicated together with 0·33 μc of ^{14}C-labelled *Chlorella*-protein hydrolysate/ml. Incubations were for 2 hr. at 30°.

Additions	Radioactivity of protein (mμc/g.)	Inhibition (%)
None	257	
Penicillin (100 units/ml.)	245	None
Penicillin + chloramphenicol (10 μg./ml.)	108	58
Penicillin + puromycin (10 μg./ml.)	34	87
Penicillin + streptomycin (10 μg./ml.)	118	54
Penicillin + ristomycin (10 μg./ml.)	285	None
Penicillin + vanomycin (10 μg./ml.)	270	None
Penicillin + novobiocin (10 μg./ml.)	266	None

osmotic 'shock' and cells exposed to osmotic 'shock' in the presence of ribonuclease were compared. There was no difference between the ribosomes of normal and 'shocked' cells but cells subjected to osmotic 'shock' in the presence of 400 μg. of ribonuclease/ml. had lost almost all their cytoplasmic ribosomes (Plates 1–3). The mitochondria of cells 'shocked' in the absence of ribonuclease were relatively unaltered. Some were swollen and some seemed to be unusually condensed but none were seen to be obviously ruptured. The mitochondria of cells 'shocked' in the presence of 400 μg. of ribonuclease/ml. were more abnormal. Whereas the mitochondria of normal Krebs cells show no sign of the presence of internal ribosome-like structures, nearly all the mitochondria of cells 'shocked' in the presence of ribonuclease did show such structures. It may well be therefore that all mitochondria do contain ribosomes, as suggested by the electron micrographs of André & Marinozzi (1965), but that in Krebs cells these only become visible when the mitochondria are swollen.

Bacterial contamination as a source of apparent synthesis. As a final check that incorporation of radioactive amino acids into mitochondrial protein during incubation *in vitro* was not due, as suggested by Decken *et al.* (1966), to bacterial contamination, we incubated isolated mitochondria with various

antibiotics. The mitochondria were isolated and washed with sucrose–EDTA–nicotinamide in the usual way, then divided into eight equal portions and incubated as indicated in Table 6. As shown in Table 6, a mixture of penicillin and ristomycin, penicillin and vanomycin or penicillin and novobiocin had no measurable effect on amino acid incorporation. Streptomycin did inhibit protein synthesis but this compound caused extensive clumping of mitochondria. As might be expected, puromycin and chloramphenicol both produced strong inhibition of amino acid incorporation into protein, but these compounds are well-known inhibitors of protein synthesis and their effect was expected irrespective of the presence of bacteria.

DISCUSSION

The experiments reported in this paper show that ribonuclease can be introduced into the cytoplasm of Krebs cells without killing the cells. This ribonuclease inhibits selectively the protein synthesis that normally occurs on the ribosomes. Since the labelling of mitochondrial proteins is not inhibited to the same extent, it seems probable that at least some of the mitochondrial proteins are synthesized independently of the microsome system. In previous investigations from this Laboratory we established that, when isolated mitochondria were incubated in buffered succinate plus a radioactive amino acid, the insoluble proteins of the mitochondrion became highly radioactive but the soluble proteins were relatively lightly labelled. Indeed, two of these that were isolated in a pure state were not labelled at all (Roodyn, Reis & Work, 1961; Roodyn *et al.* 1962; Roodyn, 1962). These results taken together with those of the present paper argue strongly for the view that the soluble proteins of the mitochondrion are supplied by the microsome–cell-sap system, but that at least some of the insoluble proteins are synthesized by a separate intramitochondrial protein-synthesizing system. It might be objected that Krebs cells treated with ribonuclease during osmotic 'shock' are abnormal cells, but in fact these cells can still replicate at the normal rate in the mouse peritoneum. The assumption of the existence of two separate sites of protein synthesis is borne out by the results of 'pulse'-labelling experiments with normal Krebs cells that have not been subjected to any trauma. The striking fall in the radioactivity of the microsome fraction and the corresponding rise in the radioactivity of the cell-sap fraction after a 'pulse' of [^{14}C]valine followed by a non-radioactive 'chase' has been clearly established in numerous investigations to be due to the completion and release after addition of a 'chase' of labelled peptide chains attached to the ribosomes. Were all mitochondrial proteins synthesized by the microsome–cell-sap system, the kinetics of labelling of mitochondria should mirror that of cell sap. In fact, it mirrors neither the kinetics of labelling of cell sap nor that of the microsomes. We conclude therefore that the most satisfactory explanation, as already suggested, is the existence of a separate protein-synthesizing system inside the mitochondria specifically devoted to the synthesis of some of the insoluble mitochondrial proteins.

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