BBA 46206

PATHWAYS OF INTRACELLULAR HYDROGEN TRANSPORT IN THE WALKER CARCINOSARCOMA 256

II. OBSERVATIONS ON OXIDOREDUCTION CHANGES OF ELECTRON CARRIERS IN SLICES

A. CITTADINI*, T. GALEOTTI, M. RUSSO** AND T. TERRANOVA
*Institute of General Pathology, Catholic University****, Rome (Italy)*
(Received May 14th, 1971)

SUMMARY

1. Addition of cyanide to slices of Walker carcinosarcoma 256, incubated in an oxygenated medium and utilizing endogenous substrates, causes the transition of cytochrome *b* from an oxidized to a reduced steady state. A further reversible reduction of 20–40 % occurs upon changing the gas phase from O_2 to N_2.

2. Nicotinamide adenine dinucleotides and flavoproteins (but not cytochrome $c + c_1$ or $a + a_3$) also undergo reversible redox changes during normoxia–anoxia cycles in the presence of cyanide.

3. The extra reduction of cytochrome *b* and flavoproteins induced by anaerobiosis in the presence of cyanide is reversed by pyruvate.

4. Electron microscopic examination of the Walker tumour shows the presence of a considerable amount of Golgi membranes but a very low content in smooth endoplasmic reticulum.

5. Spectrophotometric analysis of the microsomal fraction of the tumour indicates the presence of cytochrome b_5 and another, unidentified *b*-type pigment (peaks at 559, 530 and 427 nm), whereas cytochrome P-450 is lacking.

6. The cyanide-insensitive redox changes of electron carriers described above are attributed to an electron transport system involving the cytochrome *b*-type pigments of the microsomal fraction.

INTRODUCTION

In the accompanying paper[1], we suggest that the relative redox potentials of intracellular compartments may be of importance in the control of energy metabolism in tumour cells. One factor involved in the redox control of the cytosolic compartment may be an electron transfer chain in the microsomal fraction. Such systems have been much studied in normal mammalian cells, but not in tumours.

* Present address: Johnson Research Foundation, University of Pennsylvania, Philadelphia Pa. 19104, U.S.A.
** Present address: Institute of General Pathology, University of Rome, Rome, Italy.
*** Postal address: via Pineta Sacchetti 644, 00168 Roma, Italy.

In this paper we have followed the changes in the redox state of cytochromes, flavins and pyridine nucleotides that occur during normoxia–anoxia transitions of slices of Walker carcinosarcoma 256 whose mitochondrial electron transport has been previously inhibited with cyanide. The results obtained indicate the existence of electron transport pathway(s) which seem to take place in membrane system(s) different from the inner mitochondrial membrane and may resemble the mixed-function oxidase systems of normal mammalian cells.

MATERIALS AND METHODS

Growth conditions, characteristics and preparation of slices of Walker carcinosarcoma 256 are described in the preceeding paper[1].

The microsomal fraction was prepared either by the method of OMURA AND SATO[2] or by that of REMMER *et al.*[3]. The final microsomal pellet was suspended at a protein concentration of about 20 mg/ml in 0.15 M KCl, containing 50 mM, pH 7.5 Tris–HCl buffer. Cytochrome b_5-depleted microsomes were prepared by the method of NISHIBAYASHI AND SATO[4]. The microsomal suspensions were kept at 4° and used on the day of preparation. The microsomal preparations were virtually free of any haemoglobin contamination as judged by difference absorption spectra of microsomes saturated with CO *vs.* aerobic microsomes.

Redox changes of electron carriers in slices were followed with the Dual wavelength/split beam Aminco–Chance spectrophotometer. Each slice was mounted in a special water-tight chamber with quartz windows, which was placed in the cuvette holder of the spectrophotometer. The slice was continuously bathed with bicarbonate Ringer[5] at a constant flow rate of 3.75 ml/min. The flow was maintained by an Unita I perfusion apparatus (Braun, Melsungen) equipped with a 50-ml syringe, connected to the chamber by two plastic leads of 1 mm internal diameter. In this way both the sides of the slice were bathed and the medium leaving the chamber was drained away through two further tubes of the same diameter. The medium was equilibrated with an O_2–CO_2–CO (91.5:5.0:3.5, by vol.) mixture[6]; and O_2 was replaced by N_2, when indicated. 3.5 vol. %CO was included in the gas mixture to eliminate spectral interferences due to the small amount of haemoglobin present in the slices, without affecting the redox state of cytochrome oxidase or other extramitochondrial electron carriers[6]. At each point indicated in the figures, the 50-ml syringe containing medium was quickly replaced by another one in which the medium was either equilibrated with another gas mixture to be tested, or contained other appropriate additions. The average thickness of the slices was roughly determined from the weight of the slice and of a piece of graph paper corresponding to its image.

Difference absorption spectra of the microsomal fraction were measured in the Dual wavelength/split beam Aminco–Chance spectrophotometer with cuvettes of 1 cm optical path. Cytochrome b_5 content was determined using $\Delta\varepsilon_{(424-409\ nm)} = 165\ cm^{-1} \cdot mM^{-1}$ (ref. 7). Electron micrographs were taken in a Philips-300 Instrument. Proteins were determined by the biuret method[8]. All the experiments were performed at room temperature.

Chemicals were purchased from Sigma (St. Louis) or E. Merck (Darmstadt).

RESULTS

Redox changes of electron carriers induced by cyanide and anoxia in slices

Fig. 1 shows the effect of cyanide and anoxia on the redox level of cytochrome *b*-type pigments, measured at 425 − 410 nm, in a slice of Walker carcinosarcoma 256. Addition of 1 mM cyanide causes an abrupt reduction of the pigment, which reaches its maximum in about 2 min. Change from O_2 to N_2 induces a further reduction of the *b* pigment, corresponding to about 40 % of that caused by cyanide. The half-time of the reduction induced by N_2 is much longer than that given by cyanide, suggesting that different electron transfer systems may be involved. The change induced by nitrogen is reversed on reoxygenation of the slice.

Fig. 2 shows measurements of cytochrome *b* (562–575 nm), $c + c_1$ (550–540 nm)

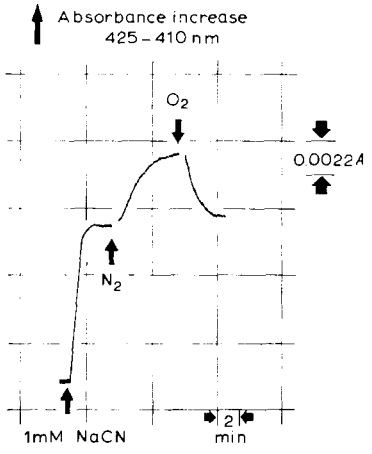

Fig. 1. Effect of cyanide and normoxia–anoxia transition on the redox level of *b*-type cytochromes in a slice of Walker carcinosarcoma 256. The slice, 0.55 mm thick, was bathed with a continuous flow of bicarbonate Ringer (for composition, see MATERIALS AND METHODS) and the redox changes measured spectrophotometrically with the dual wavelength technique[9] at 425–410 nm. After incubation of the slice for about 10 min in the medium equilibrated with an O_2–CO_2–CO (91.5:5.0:3.5, by vol.) mixture, 1 mM NaCN was added. At the points indicated, the gas was changed from O_2–CO_2–CO to N_2–CO_2–CO and *vice versa*. Endogenous substrate.

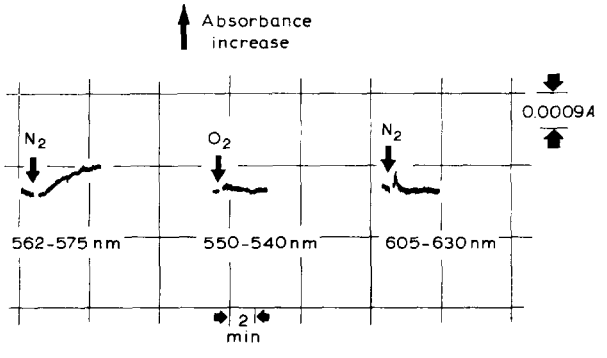

Fig. 2. Effect of normoxia–anoxia transitions on the redox state of cytochrome *b* (562–575 nm), cytochrome $c + c_1$ (550–540 nm) and $a + a_3$ (605–630 nm) in a slice treated with 1 mM cyanide. Slice thickness, 1.06 mm. For other conditions, see Fig. 1.

and cytochrome oxidase (605–630 nm) made sequentially in a single slice that had been treated aerobically with 1 mM cyanide in order to block electron transport through the mitochondrial respiratory chain. Changing the gas phase to N_2 caused a similar reduction of cytochrome *b* (α band) to that seen in Fig. 1 in the Soret region. The wavelength pair was then changed to 550–540 nm while the slice was still anaerobic; upon restoration of aerobiosis, no change was observed in the cytochrome $c + c_1$ absorption. Similarly, cytochrome $a + a_3$ showed no change when anoxia was once again induced. In contrast, nicotinamide-adenine dinucleotides (340–375 nm) and flavoproteins (460–495 nm)* showed a normoxia–anoxia cycle in the presence of cyanide similar to that given by cytochrome *b* (Fig. 3).

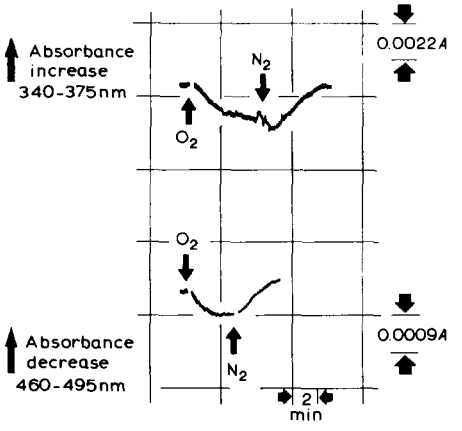

Fig. 3. Oxidation–reduction changes of nicotinamide-adenine dinucleotides (340–375 nm) and flavoproteins (460–495 nm) during a normoxia–anoxia transition in a slice (0.58 mm thick) pretreated with 1 mM cyanide. Other conditions are described in Fig. 1.

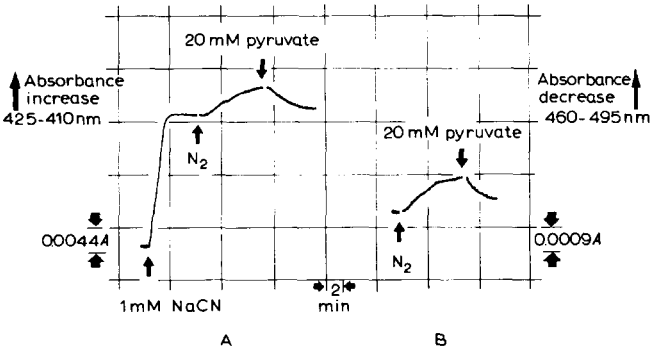

Fig. 4. Effect of pyruvate on the nitrogen-induced reduction of a *b*-type cytochrome and flavoproteins in slices treated with cyanide. Trace A, an aerobic slice (0.98 mm thick) was treated with 1 mM cyanide followed by replacement of O_2 with N_2. The nitrogen-induced reduction was partly reversed by 20 mM pyruvate. Trace B, pyruvate addition to an anaerobic slice (1.3 mm thick) treated with cyanide caused partial reoxidation of flavoproteins reduced by N_2. For other conditions, see Fig. 1.

* It should be noted that the flavoproteins measured here by absorption appear to be extramitochondrial, since no absorbing flavoproteins were detected in isolated mitochondria (ref. 1).

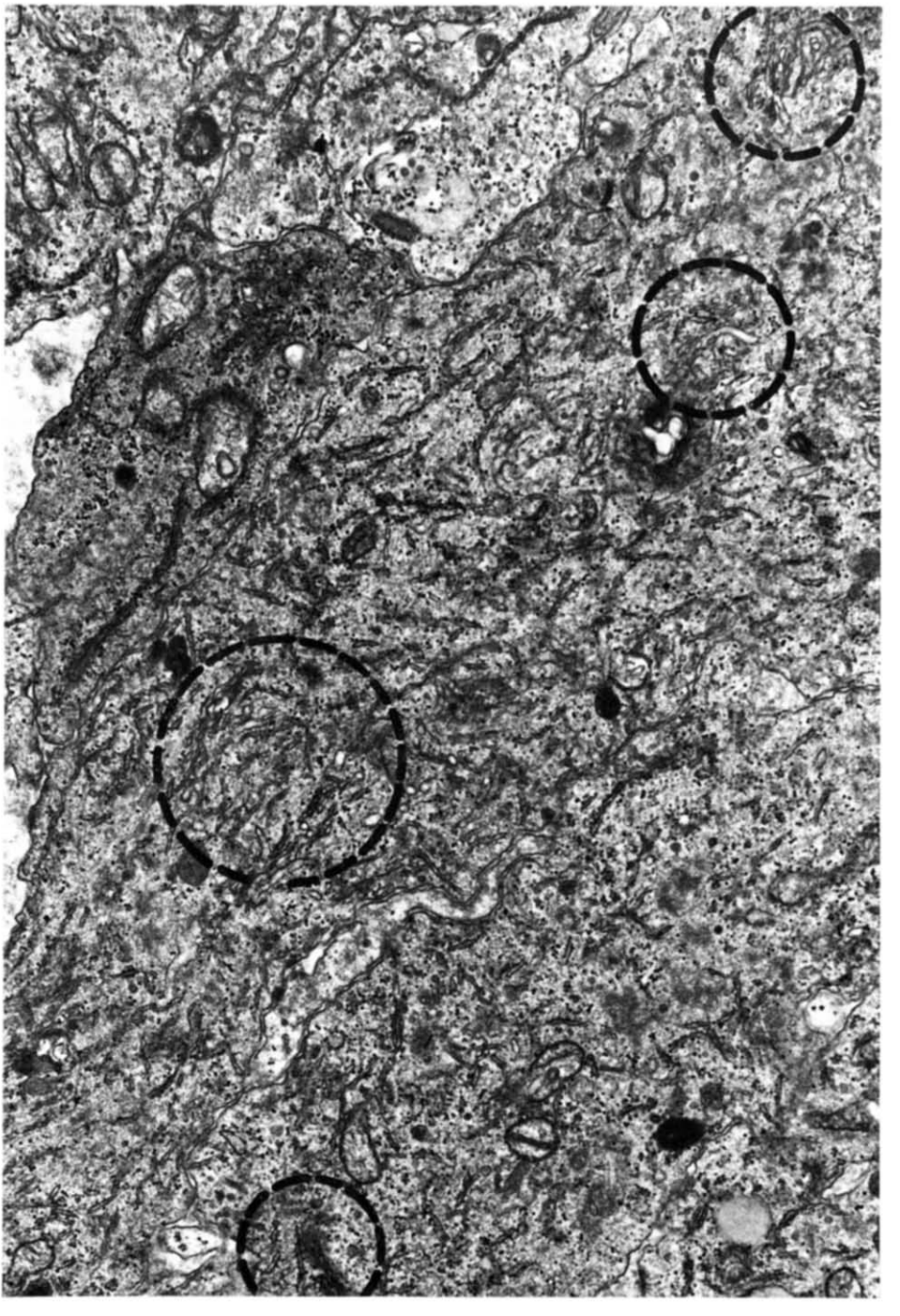

Fig. 5. Electron micrograph of Walker carcinosarcoma 256. Fixation in 2.5% glutaraldehyde followed by 1.33% osmium; staining with 1% lead hydroxide. The presence of several Golgi apparatuses, marked in the figure, is evident. ×17000.

Effect of pyruvate on the redox level of cytochrome b and flavoproteins in anaerobic slices treated with cyanide

Fig. 4 shows redox changes of cytochrome *b* and flavoproteins done separately in two slices of different thickness. The effects of cyanide and anaerobiosis on cytochrome *b* are similar to those observed in Fig. 1. Addition of 20 mM pyruvate, under anaerobic conditions, causes a reoxidation of almost all the cytochrome reduced by anaerobiosis, but not of that reduced by cyanide (Fig. 4A). Pyruvate addition also reverses partially the reduction of flavoproteins induced by anaerobiosis (Fig. 4B).

Electron microscope examination of the intact tissue

From the data presented above it appears possible to use combinations of cyanide and O_2/N_2 transitions in order to distinguish the response of respiratory carriers in the intra- and extramitochondrial space of the cell. Electron micrographs of the tumour have been taken in an attempt to obtain evidence for the presence of membrane systems, other than the mitochondria, in which an electron transport involving NAD(P)H, flavoproteins and a cytochrome *b*-type pigment could occur. As shown in Fig. 5, the cells appear to be almost completely free of smooth endoplasmic reticulum, but Golgi membranes are quite well represented.

Spectrophotometric detection of cytochromes in the microsomal fraction

In an attempt to obtain further evidence for the possible site of cyanide-in-

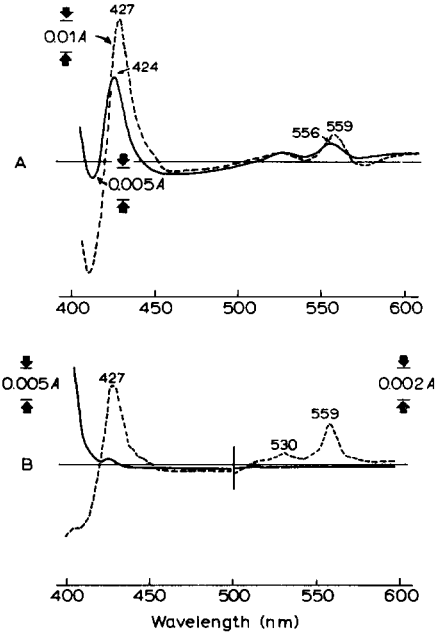

Fig. 6. Difference absorption spectra of normal (A) and b_5-depleted (B) microsomal preparations of Walker carcinosarcoma 256. Spectra were recorded from NADPH-treated *minus* untreated aerobic microsomes (——) and (NADPH *plus* $Na_2S_2O_4$)-treated *minus* NADPH-treated aerobic microsomes (- - - -). The microsomes were suspended in 0.1 M Tris–HCl buffer, pH 7.5 at the concentration of 3.2 mg protein/ml (A) and in 0.1 M Tris–HCl buffer, pH 7.4, containing 30% glycerol, at the concentration of 1.5 mg protein/ml (B). NADPH was added at the final concentration of 330 μM.

sensitive electron transfer systems, room-temperature difference spectra were performed on the microsomal fraction isolated from the Walker tumour (Fig. 6). Addition of NADPH to aerobic microsomes reduces cytochrome b_5 (α-band at 556 nm, Soret band at 424 nm). The amount of cytochrome b_5 reducible by NADPH in these preparations is 0.02 nmole/mg protein (average of four experiments). Subsequent addition of dithionite causes reduction of another *b*-type cytochrome with absorption maxima at 559, 530 and 427 nm for the α, β-, and γ-bands, respectively. If the microsomes are depleted of cytochrome b_5 (Fig. 6B), the addition of dithionite is still able to reduce this unknown pigment. Treatment of microsomes with carbon monoxide does not cause a shift or enhancement of the γ-band of the dithionite-induced pigment. As indicated by difference absorption spectra (not shown) of microsomal samples treated with CO *plus* dithionite *vs.* samples reduced with dithionite, only a low amount of cytochrome P-420 and no cytochrome P-450 was detected.

DISCUSSION

In the accompanying paper[1] we have shown that in slices of Walker carcinosarcoma 256 respiration is not completely blocked by site-specific inhibitors of the mitochondrial electron transport. About 15 % of the basal rate of O_2 consumption appears to be insensitive to these inhibitors. Similar inhibitor-insensitive respiration has been observed in rat-liver preparations[10–13]. Oxygen uptake by isolated mitochondria is completely blocked by such inhibitors, and the inhibitor-insensitive respiration of whole cells has therefore been attributed to extramitochondrial electron transfer pathways[13–18].

These observations have prompted us to investigate further the insensitive respiration of tumour slices by following the oxidoreduction changes of respiratory pigments with the double-beam technique[9]. Under the conditions used, replacement of O_2 with N_2 and *vice versa* in the gas mixture should only produce changes in the redox level of respiratory pigments related to electron transport activity occuring in the extramitochondrial compartment of the cell. Indeed, as shown in Figs. 1 and 2, addition of N_2 to cyanide-inhibited slices causes an increase in the redox level of a cytochrome *b*-like pigment, which is reversed by reoxygenation of the slice, but no changes of cytochrome $c + c_1$ and $a + a_3$. The reason we have chosen the wavelength pair 425–410 nm for the γ-band, instead of 430–410 nm, was that we anticipated detection of larger redox changes of *b*-type cytochromes like cytochrome b_5 which have an absorption maximum shifted by about 5 nm towards the ultraviolet region with respect to cytochrome *b*.

These data allow us to draw the following conclusions about the absorption changes induced by the O_2/N_2 transition: (a) haemoglobin does not seem to interfere with these measurements; (b) the possibility that the effect of anaerobiosis is to block completely a leak of electrons in the mitochondrial respiratory chain still existing in the presence of cyanide may be ruled out and finally (c) the cytochrome *b*-like pigment we are dealing with is different from the cytochrome *b* of the mitochondrial respiratory chain. The finding that the cytochrome *b* kinetics are accompanied by similar changes in the redox level of nicotinamide-adenine dinucleotides and flavoproteins (see Fig. 3) suggests that the proposed extramitochondrial electron

transport sytem involves these three types of electron carrier. The effect of pyruvate addition in reoxidizing a large portion of cytochrome *b* and flavoproteins reduced by anoxia may be explained in terms of competition for the oxidation of cytosolic NADH between the lactate dehydrogenase reaction and the cyanide-insensitive electron transfer pathway.

In mammalian tissues, such as rat liver or adrenal cortex the cytosolic redox potential of NAD(P)H may be controlled by the oxidase activity associated with membrane systems located in the extramitochondrial space of the cell[16-18]. Mixed-function oxidation linked to the metabolism of the drugs and steroids in the endoplasmic reticulum[19-21] or peroxidatic reactions in the peroxisomes (microbodies)[22] could well contribute to the reoxidation of reducing equivalents generated in the cytosol. Thus the antimycin A- and cyanide-insensitive respiration of rat-liver slices[10,23] or perfused liver[11-13] has been correlated with such extramitochondrial activities[13-18]. In the tumour cells the rather low content of such membrane structures, as displayed by electron microscopy and by measurements of the specific activities related to them (see ref. 24 for review), might bring into question the possible role of these extramitochondrial functions in the control of the cytosolic $NAD(P)^+$ redox state. However, more recently it has been found that other membrane systems of liver cells contain electron carriers and enzymatic activities which were previously assigned only to one type of membrane. Cytochrome b_5, for instance, has been found to be present not only in the smooth- and rough-surfaced microsomes but also in the Golgi apparatus[25,26] and nuclei[26,27]. NADPH–cytochrome *c* reductase and NADH–cytochrome b_5 reductase have also been detected in Golgi vescicles[25,28,29]. The Walker carcinosarcoma 256 is a tumour which typically shows a high concentration of free ribosomes, as single units or aggregates (polysomes), with a small amount of rough- and practically no smooth-surfaced endoplasmic reticulum. However, the Golgi apparatus, as it has been reported for other tumours[30], is rather abundant and the nucleus occupies a large part of the cell volume. The whole microsomal fraction isolated from the Walker tumour contains cytochrome b_5 and no cytochrome P-450. Since this fraction consists not only of smooth and rough microsomes but also of plasma membranes and Golgi vescicles, the small but significant content of cytochrome b_5 that we find in the microsomal fraction of the Walker tumour may derive mainly from the contamination of such fraction by Golgi membranes. Indeed, these membranes, purified from rat liver, show the presence of cytochrome b_5 and very low cytochrome P-450 (ref. 25, 26)

Another cytochrome *b*-type pigment, reducible by dithionite but not by NAD(P)H in the presence of oxygen, is present in larger quantity than cytochrome b_5 in the microsomal fraction of the tumour. This pigment has the same absorbing peaks as reduced cytochrome P-420 (ref. 31), but its reduced form does not react with CO. Thus the nature of this pigment is still unknown and its identification and possible function requires further investigation.

ACKNOWLEDGEMENTS

We are grateful to Drs. B. Chance and G. van Rossum for helpful criticism of the manuscript. The work was in part supported by a grant from Consiglio Nazionale delle Ricerche, Italy.

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