Biochemical Pharmacology, Vol 24, pp. 1491–1494 Pergamon Press, 1975. Printed in Great Britain.

# INFLUENCE OF *O*-(β-HYDROXYETHYL)-RUTOSIDE OR VENORUTON ON LACTATE DEHYDROGENASE OF HUMAN CULTURED VARICOSE VEINS

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(*Received* 1 *August* 1974; *accepted* 13 *February* 1975)

**Abstract**—Lactate dehydrogenase (LDH) level and isozyme distribution are indices of the importance of glycolysis and of the relative contributions of the anaerobic and aerobic metabolism to the energy requirements of striated and smooth muscles. We therefore compared the LDH isozymic distribution of human normal and varicose saphenous veins and examined the LDH activity of varicose veins cultured without and with a flavonoid, *O*-(β-hydroxethyl)- rutoside or Venoruton. The isozymic distribution of normal and varicose veins does not differ. Varicose vein explants cultured for up to 3 days at 37° remain in fairly good condition judging from the LDH specific activity and the isozymic distribution of the controls. Venoruton influences the distribution of the LDH isozymes in a way which, according to Kaplan's theory, reduces glycolysis and could therefore favor the aerobic metabolism of the varicose vein and increase its cellular ATP level.

The relative contributions of glycolysis and respiration to the energy requirements of skeletal, cardiac and smooth muscular tissue vary considerably [1]. This metabolic adaptation corresponds to different levels of the various enzyme groups of constant proportions [1–3]. The activity of lactate dehydrogenase (EC 1.1.1.27, LDH) which can be considered as a key enzyme for glycolysis is therefore an index of the importance of this metabolic route. Kaplan and his colleagues [4] have shown furthermore, that its isozymic distribution also reflects the ratio of the anaerobic and aerobic capacities of the muscle, the white skeletal muscles containing mainly the $M_4$ ($LDH_5$) component and the aerobic ones like heart and bovine carotid, the $H_4$ and $H_3M$ ($LDH_1$ and $LDH_2$) components [3]. The percentages of the isozymes depend not only on the muscle type but also vary according to its condition. In myopathies, higher proportions of $LDH_1$ and $LDH_2$ and lower ones of $LDH_5$ are found suggesting a reversion to the foetal type [5]. Training can also shift the distribution of the isozymes in favour of the H subunit [2, 6]. Such changes may occur rapidly as the apparent half-lives of skeletal and cardiac muscle LDHs seem to be of the order of one [7] or several days [8].

Information on LDH in the vascular wall is fairly limited. LDH activity of human varicose veins seems to increase with the varicosity [9]. Shifts in isozyme distribution in human aorta containing atherosclerotic plaques and in the rat aorta after thyroxine or methylthiouracil treatment have been described [10]. Recently, we have shown by starch-gel electrophoresis that the LDH patterns of human normal and varicose veins do not seem to differ but that the treatment of the varicose vein with Venoruton appears to influence the isozyme distribution: the fast $LDH_1$ band became apparent while the intensity of the slow $LDH_5$ band decreased [11].

In this work, the LDH isozymic distribution of normal and varicose veins was further examined using agar gel electrophoresis and the effect of *O*-(β-hydroxyethyl-rutoside) on the LDH level and isozyme distribution of cultured varicose veins was investigated.

## MATERIALS AND METHODS

Normal human saphenous veins were taken at autopsy within 6 hr after death. The varicose veins were removed by surgical stripping of the internal saphenous vein. After excision, the vessels were dipped immediately into a tyrode solution at about 4° before extraction or in Hank's sterile solution for organ culture. The samples were then rinsed several times and freed of connective tissue and adventitia. To examine the influence of Venoruton on LDH, each vessel was cut in fragments about 0·5 cm long; the fragments with odd numbers and the even-numbered ones were pooled separately in sterile Petri dishes containing the normal culture medium (odd-numbered fragments) or culture medium supplemented with 3 mg/ml of Venoruton (even-numbered fragments). The culture medium was that utilised for bovine veins by Zwillenberg *et al.* [12]. It was Eagle's Minimal Essential Medium with 20% foetal bovine serum, 5 mM glutamine and 20 mM HEPES buffer; and contained 100 i.u./ml penicillin, 100 i.u./ml streptomycin, 50 μg/ml gentamycin and 5 μg/ml amphotericin B to prevent microbial growth. The pH was adjusted to 7·3 with sterile 1N NaOH. The Petri

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dishes were placed in the water-saturated atmosphere of an exsicator at 37°.

*Materials.* All chemicals used were of analytical grade and were purchased from Boehringer (Mannheim) and Sigma (St Louis, Mo). The constituents of the culture medium were obtained from Flow (Irvine, Scotland). *O*-(β-Hydroxethyl)-rutoside (Venoruton) was a gift from Zyma Ltd., Nyon, Switzerland.

*Muscle extracts.* The pieces of vein were blotted gently with filter paper, cut with scissors and homogenized at 4° with a Polytron 10 ST-OD homogenizer in 5 vol 50 mM phosphate buffer at pH 7·5 for determination of enzyme sp. act., or in 2 vol isoosmotic glycerol (about 2%) containing 2 mM EDTA and 10 mM Tris at pH 8·6 [13] for agar gel electrophoresis. In the latter case, the supernatant obtained after centrifugation was brought to pH 5 with 1N acetic acid, the insoluble matter was removed and the solution was immediately readjusted to pH 7·5 with 2 M Tris [3]. It was then concentrated approximately 5 times using Minicon B-15 microconcentrators (Amicon Ltd). Protein content was determined by the biuret method [14]; the interference by Tris of the colour development [15] was taken into account. Total LDH activity of the phosphate extract was measured according to Bergmeyer *et al.* [16] with 1 mM pyruvate and 0·1 mM NADH final concentrations.

*Agar gel electrophoresis.* This method which gives a better separation of the LDH isozymes than starch-gel electrophoresis, was carried out according to Wieme [17]. The 12 × 5 cm glass slides were coated with a 1 mm thick layer of a 1% Difco Special Agar Noble in 0·03 M barbital buffer at pH 8·4. Two 14 mm wide slits separated by a 10 mm interval were made on each gel; they were filled with 15 μl of a 1% protein solution. The run was conducted at 4° for 75 min with a constant current intensity of 1·5 mA/cm and a voltage of about 200 V. The LDH bands were revealed by positive staining according to Van der Helm [18]. The percentages of the LDH isozymes were evaluated from densitometer traces at 570 nm given by a Quick scan electrophoresis densitometer provided with a built-in integrator (Helena Lab., Beaumont, Texas), the sum of integrating impulses of the five isozymes being expressed as 100%.

The results were analysed statistically using the Student's *t*-test; a difference corresponding to the probability coefficient $P < 0{\cdot}05$ was taken as significant.

## RESULTS

The apparently similar distribution of the LDH isozymes in normal and varicose veins suggested by starch gel electrophoresis [2] has been reexamined on agar gels. Each electrophoresis was carried out on two extracts corresponding respectively to a normal and to a varicose vein (Fig. 1a and b). The fast $H_4$ band is obvious in both patterns on the slide, but hardly visible on the print. The percentages evaluated by densitometry are given in Table 1. They are fairly

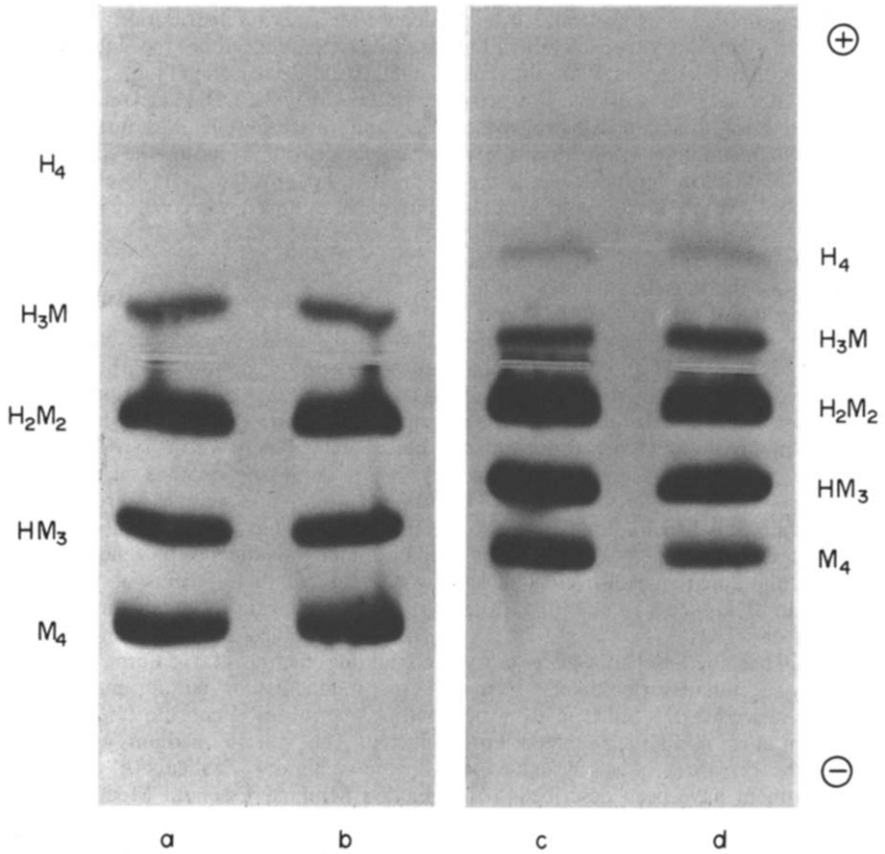


Fig. 1. Agar gel electrophoresis patterns of the LDH isoenzymes of human vein extracts. *Left*: normal vein (a) and varicose vein (b). *Right*: fragments of the same varicose vein cultured without (c) and with Venoruton (d).

Table 1. Mean percentages ± S.E. of the LDH isozymes of normal and varicose veins determined by densitometry of agar gel patterns

| Isozyme | Normal veins (n* = 6) | Varicose veins (n* = 6) | P† |
|---|---|---|---|
| $H_4(LDH_1)$ | 1 ± 0·1 | 1·2 ± 0·1 | <0·3 |
| $H_3M(LDH_2)$ | 8·7 ± 0·9 | 9·3 ± 0·9 | <0·6 |
| $H_2M_2(LDH_3)$ | 32·2 ± 0·8 | 30·8 ± 0·8 | <0·3 |
| $HM_3(LDH_4)$ | 30·3 ± 1·2 | 30·2 ± 1·2 | <1 |
| $M_4(LDH_5)$ | 27·8 ± 0·8 | 28·5 ± 1 | <1 |

* Number of veins examined.
† Probability coefficient of the Student's *t*-test.

constant from one vein to the other within each set and the isozymic distribution is unaltered in disease.

In view of this result and of the difficulties encountered in getting satisfactory normal material, subsequent work was carried out on cultured varicose veins. The change in the LDH specific activity over the culture period of 6 days is shown in Table 2. The activity decreases slowly in controls. In the presence of Venoruton, a larger decrease was observed which is significant after 1 and 3 days; after 6 days of culture, the result was less clearcut but, as degeneration of the muscle cells sets in after 4 days under these conditions [12], this last figure is less valid.

The isozymic distributions of vein explants cultured for 3 days without or with Venoruton were also compared (Fig. 1c and d). It is obvious that the slow $M_4$ band decreased in the vein explant kept alive in the presence of Venoruton. This decrease was very reproducible. The densitometric analysis of Fig. 1 (Table 3) shows that the $^{H}/_{M}$ ratio of the explants cultured without Venoruton does not differ from that of varicose veins while a shift occurs under the influence of Venoruton: the percentage of the H subunit increases from 35 to 40%. As the probability coefficient P is <0·05, this increase is significant. As the experiments have been carried out in parallel on two comparative samples of the same vessel, the results have also been analysed statistically by the paired *t*-test; the decrease of $M_4$ and the increase of $M_2H_2$ are significant.

It is possible that a shorter culture period could bring about the same change as degeneration of the muscle cells may occur within 6 days. In view of the restricted variation found, the evolution of the pattern in the course of the culture has not been examined.

## DISCUSSION

The examination of the activity levels of several enzymes of dog carotid has suggested that vertebrate smooth muscle has the enzymatic activity profile typical of aerobic muscles [1]. LDH isozymic patterns corresponding to various vessels show, however, that the isozyme distribution corresponds to that of white or red muscle according to the species or location [10, 19]. The picture arising from measurements of oxygen consumption and lactic acid production is also equivocal. In the presence of glucose or succinate, the relative contributions of oxidation and glycolysis to glucose degradation in the case of bovine tibialis vein amount to 16 and 84%, respectively [20, 21]. Very different results can be obtained if glucose or fatty acids are added to the incubation medium of the ox aorta in view of the large share of energy coming, in this case, from fatty acid oxidation [22]. With this background, it is difficult to draw a definite conclusion from our results showing the same percentages of the LDH isozymes in normal and varicose veins. They suggest that lower oxygen consumption in the presence of glucose or succinate found in varicose veins [20, 21] might not reflect the true oxygen consumption of the tissue *in vivo* and that such determinations should be carried out also in the presence of fatty acids. Our data suggest that the higher anaerobicity of varicose veins due to blood stasis does not influence the LDH isozyme distribution and is in favor of an unchanged aerobicity of the muscle cells of the diseased saphenous veins.

The unavoidable drawbacks bound to the utilization of human autopsy material could be avoided in the case of varicose veins where two as similar as possible samples from the same vein are compared. At 37°, the drop in LDH activity of the control amounts after 6 days to only 25% of the figure obtained after 1 day. The cultures remain, therefore, in fairly good condition after such a period, supporting earlier observations [12]. The two effects of Venoruton, a decrease of LDH specific activity and the increase of relative percentage of the H subunit, although not very large, are significant as shown by

Table 2. LDH specific activities (U/mg protein) of varicose veins* cultured without or with Venoruton (3 mg/ml)

| | After 1 day | After 3 days | After 6 days |
|---|---|---|---|
| Untreated | 0·988 ± 0·15 | 0·875 ± 0·12 | 0·754 ± 0·14 |
| Treated | 0·855 ± 0·11 | 0·681 ± 0·12 | 0·558 ± 0·13 |
| Decrease (in %) | 14 | 22 | 26 |
| P† | <0·05 | <0·05 | 0·05 < P < 0·1 |

* Number of veins examined = 5.
† Coefficient of probability determined by the comparison of two series by the paired *t*-test.

Table 3. Mean percentages ± S.E. of the LDH isozymes of varicose veins cultured without or with Venoruton (3 mg/ml) determined by densitometry of agar gel patterns

| Isozyme | Controls (n* = 6) | Treated veins (n* = 6) | P† |
|---|---|---|---|
| $H_4(LDH_1)$ | 1·1 ± 0·2 | 1·4 ± 0·3 | < 0·5 |
| $H_3M(LDH_2)$ | 10·3 ± 2 | 14·0 ± 3·5 | < 0·2 |
| $H_2M_2(LDH_3)$ | 39·0 ± 4·6 | 43·5 ± 5 | < 0·02 |
| $HM_3(LDH_4)$ | 27·0 ± 2 | 25·1 ± 4·5 | < 0·2 |
| $M_4(LDH_5)$ | 22·6 ± 5 | 16·0 ± 4·7 | < 0·01 |

* Number of veins examined.

† Coefficient probability determined by the comparison of two series by the paired *t*-test.

the statistical analysis and by visual observation of the patterns of the six varicose veins examined. As the culture cannot be prolonged further under our experimental conditions, a more extensive change may be difficult to achieve *in vitro*.

In conclusion, Venoruton does not act only by modifying enzymic activities of the vascular wall [22]; it effects the distribution of the LDH subunits in a way which reduces glycolysis and could therefore favor the aerobic metabolism of the varicose vein and increase its cellular ATP level.

*Acknowledgements*—The authors wish to thank Dr. J. Collin, and Prof. H. Betz, director of the Department of Pathological Anatomy of the University, for their precious help in the collection of the experimental material. They are grateful to Zyma-Galen, Brussels, for generous financial support and to Dr. L. O. Zwillenberg of the Zwillenberg Tietz Laboratorium, Bern, for advice on culture problems.

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