produced formaldehyde nor glycolaldehyde on periodate oxidation. If sulphate groups had been esterified at 3,6 or 3,4 carbon of *N*-carbobenzoxyglucosamine, the compound would have consumed periodate, with the production of formaldehyde in the case of 3,4-disulphate, and glycolaldehyde sulphate in the case of 3,6-disulphate[17–19]. Thus, in the present compound, the sulphate groups are esterified at 4 and 6 carbon of *N*-carbobenzoxyglucosamine.

The effect of various hexosamine sulphates on arylsulphatase B activity is shown in Table I. At 10 m*M* carbobenzoxygalactosamine 6-sulphate or galactosamine 6-sulphate has no effect on enzyme activity, whereas UDP-*N*-acetylgalactosamine 4-sulphate at 10 m*M* produces 60% inhibition of arylsulphatase B activity. Both carbobenzoxyglucosamine 4,6-disulphate and glucosamine 4,6-disulphate inhibited the enzyme activity of 50% at 10 m*M*. The nature of inhibition in all cases was a competitive one and for carbobenzoxyglucosamine 4,6-disulphate is shown in the Figure. The glucosamine 4,6-disulphate was also coupled with CH-sepharose by the method of ALLEN and NEUBERGER[20] in an attempt to make an affinity chromatography column for arylsulphatase B. The enzyme did not show any affinity for glucosamine 4,6-disulphate bound CH-sepharose under variety of conditions of pH and ionic strength.

The rate of hydrolysis of various hexosamine sulphates by arylsulphatase B is shown in Table II. There was no release of sulphate either from carbobenzoxygalactosamine 6-sulphate or galactosamine 6-sulphate. The release of sulphate was approximately 2.5 times higher in the case of UDP-*N*-acetylgalactosamine 4-sulphate compared with glucosamine 4,6-disulphate. Here it must be recalled that earlier attempts to show the presence of sulphatase acting on hexosamine sulphate have been unsucessful[21], probably because the substrates used were hexosamine 6-sulphates. The present study confirms the previous reports by showing that arylsulphatase B has no activity towards galactosamine 6-sulphate. Further it lends additional support to the suggestions[11,22,23] that the *N*-acetylgalactosamine 4-sulphate in dermatan sulphate and chondroitin 4-sulphate is a naturally occurring substrate for arylsulphatase B. The hydrolysis of glucosamine 4,6-disulphate by arylsulphatase B also indicates that the enzyme has a high specificity for the position of sulphate moiety and the nature of hexosamine moiety is not so important.

[20] A. K. ALLEN and A. NEUBERGER, FEBS Lett. *50*, 362 (1974).
[21] K. S. DODGSON and A. G. LLOYD, Biochem. J. *78*, 319 (1961).
[22] E. SHAPIRA, R. R. DEGREGORIO, R. MATALON and H. L. NADLER, Biochim. biophys. Res. Commun. *62*, 448 (1975).
[23] J. F. O'BRIEN, M. CANTZ and J. SPRANGER, Biochim. biophys. Res. Comm. *60*, 1170 (1975).

Close Correlation between Levels of Cholesterol and Free Fatty Acids in Lymphoid Cells

S. KIGOSHI, M. AKIYAMA and R. ITO

Department of Pharmacology, School of Medicine, and Department of Pharmacology, Cancer Research Institute, Kanazawa University, Kanazawa 920 (Japan), 6 April 1976.

Summary. A close correlation was found between the levels of free cholesterol and free fatty acids in lymphoid cells from thymus, spleen or lymph node of mice and guinea-pigs. This relationship suggests a possible role of cholesterol regulating the fatty acid levels in lymphoid cells.

It is well known that the structural lipid of mammalian cell membrane consists primarily of phospholipid and cholesterol, and their proportions and structure are very important to the properties and functions of cell membrane[1–4]. Free cholesterol has recently been shown to be involved in the membrane fluidity relating to the rigidity of surface membrane of lymphocytes[5]. Studies on lymphocyte lipids have revealed that the cholesterol levels in normal lymphocytes from man and animals differ markedly from that in leukemic cells[5–7]. In a previous communication we reported that growth of Ehrlich's ascitic carcinoma in mice results in increase of free cholesterol and free fatty acids in lymphoid cells from thymus, spleen and cervical lymph node, but decrease of these lipids in the cells from mesenteric lymph node[8]. The present study demonstrates a close correlation between the levels of free cholesterol and free fatty acids in lymphoid cells from thymus, spleen or lymph nodes of mice and guinea-pigs.

The thymus, spleen and lymph nodes (cervical and mesenteric lymph node) were obtained from the following groups of female mice (*ddN* strain): mice bearing with Ehrlich's ascitic carcinoma, mice bearing with solid tumors of Ehrlich's cells on tail and animals without tumors (normal mice, 24–26 g). These 3 groups of mice were fed with diet and given water ad libitum before the experiments. 1 group of mice receiving the i.p. inoculation of tumor cells (5×10^6 cells/mouse) were killed by cervical dislocation on the 5th and 10th day after inoculation (mice bearing with ascitic carcinoma)[8]. Another group of mice implanted s.c. with tumor cells midway up the tail (4×10^6 cells/mouse) was killed 7, 14 and 21 days after implantation, except the animals which showed no growth of solid tumors (mice bearing with solid tumors)[9]. The mouse tissues were also obtained from normal animals deprived of diet for 48 h before the experiments. Preparing of lymphoid cells from mouse tissues and lipid quantitation of the cells were performed by the method described previously[8,10]. In addition, lymphoid cells were prepared from spleen and inguinal lymph node of guinea-pigs (Hartley strain, 500–700 g) fed with diet ad libitum before the experiments[10]. Before lipid quantitation, the guinea-pig lymphoid cells were suspended in Krebs-Ringer phosphate buffer (pH 7.4)[11] containing 2% of bovine albumin fraction V (Armour Laboratories) and incubated at 37 °C for 2 h in the presence of adrenergic agents (isoproterenol, epinephrine or norepinephrine) at concentrations of 10^{-5}–10^{-10} *M* (drug-treated cells) or in the absence of the drugs (control cells)[12].

The contents of free cholesterol, free fatty acids and phospholipids in lymphoid cells from three groups of mice (normal mice, mice bearing with ascitic carcinoma at 10 days and animals bearing with solid tumors at 14 days) were presented in Figure 1. As can be seen, the levels of each lipid fraction in lymphoid cells from thymus, spleen

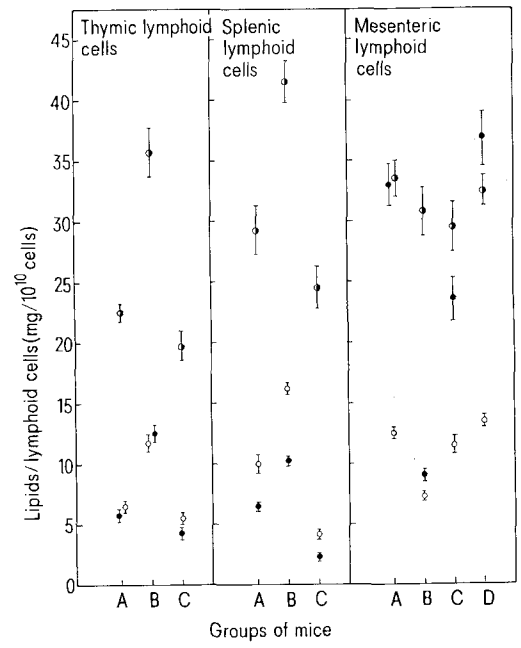

Fig. 1. Levels of free cholesterol, free fatty acids and phospholipids in lymphoid cells from thymus, spleen and mesenteric lymph node of mice. The tissues were obtained either from normal mice (group A), mice bearing with ascitic carcinoma on day 10 after inoculation (group B) or from animals bearing with solid tumors on tail 14 days after implantation (group C). These 3 groups of mice were fed with diet and given water ad libitum before the experiments. Lipid contents of mesenteric lymphoid cells from mice deprived of diet for 48 h before the experiments were also presented (group D). All values represent mean ± SE of 6 or 8 experiments.
Symbols: ○, cholesterol; ●, fatty acids; ◑, phospholipids.

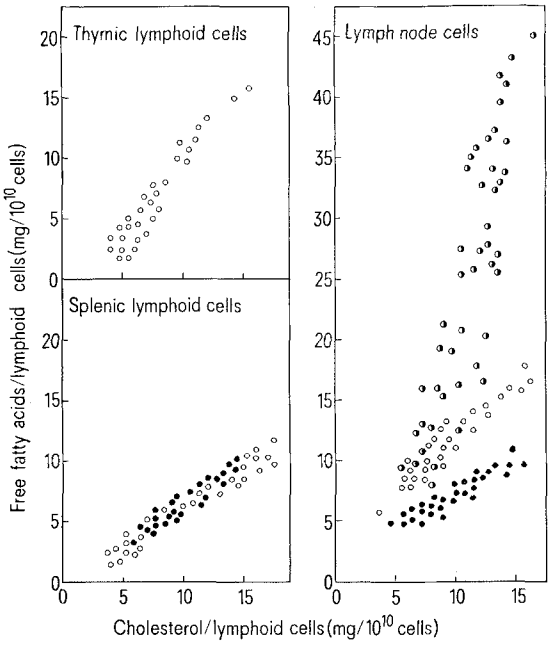

Fig. 2. Relationship between the levels of free cholesterol and free fatty acids in lymphoid cells from mice and guinea-pigs. Lymphoid cells were prepared from the following tissues: thymus, spleen, cervical lymph node and mesenteric lymph node of mice, and spleen and inguinal lymph node of guinea-pigs. Symbols: ○, lymphoid cells from mouse tissues except mesenteric lymph node; ●, lymphoid cells from guinea-pig tissues; ◑, mesenteric lymphoid cells from mice.

or mesenteric lymph node varied greatly with the groups of mice. Changes in the cholesterol levels of lymphoid cells from mice were accompanied by changes in the levels of other lipid fractions within the respective tissues, except the phospholipids in the mesenteric lymphoid cells. Each of the fatty acid values in lymphoid cells from mice was then plotted against the corresponding values of cholesterol within each of the lymphoid tissues including cervical lymph node (Figure 2). Apparently there was a nearly linear relationship between the levels of cholesterol and fatty acids in lymphoid cells within every one of the tissues of mice: The correlation coefficient of fatty acid to cholesterol was 0.94 for thymic lymphoid cells (regression coefficient: $b = 1.21$, $n = 42$), 0.99 for splenic lymphoid cells ($b = 0.65$, $n = 42$), 0.92 for mesenteric lymphoid cells ($b = 3.71$, $n = 50$) and 0.95 for cervical lymphoid cells ($b = 0.86$, $n = 32$), respectively ($p < 0.01$). On the other hand, the correlation coefficient calculated from the values of phospholipids and cholesterol in lymphoid cells from mice was 0.70 for thymus ($b = 1.64$), 0.83 for spleen ($b = 1.35$), 0.19 for mesenteric lymph node and 0.24 for cervical lymph node, respectively ($p < 0.01$ for thymic and splenic lymphoid cells). Figure 2 also shows the relationship between the levels of cholesterol and fatty acids in lymphoid cells from spleen and inguinal lymph node of guinea-pigs. The contents of each lipid fraction in guinea-pig lymphoid cells varied greatly from day to day. However, adrenergic agents had little effect on the levels of cholesterol, fatty acids and phospholipids in guinea-pig lymphoid cells (lipids in drug-treated cells/lipids in control cells: 0.91–1.23)[12]. In guinea-pigs, the correlation coefficient of fatty acid to cholesterol was 0.94 for splenic lymphoid cells ($b = 0.69$, $n = 42$) and 0.93 for inguinal lymphoid cells ($b = 0.53$, $n = 30$), respectively ($p < 0.01$), whereas the correlation coefficient of phospholipid to cholesterol for splenic and inguinal lymphoid cells from guinea-pigs was 0.54 ($b = 1.20$) and 0.56 ($b = 0.64$), respectively ($p < 0.01$). It is evident, therefore, that the relation of free fatty acid to free cholesterol in lymphoid cells from mice or guinea-pigs is more intimate than that of phospholipid to free cholesterol. Furthermore it is noticeable that the regression coefficient of fatty acid to cholesterol for splenic lymphoid cells of mice was very similar to that for the corresponding cells of guinea-pigs (0.65 for mice and 0.69 for guinea-pigs, $p < 0.01$). The correlation coefficient calculated from the values of fatty acids and cholesterol in splenic lymphoid cells from 2 animals was 0.98 ($b = 0.66$, $n = 84$) ($p < 0.01$).

The present results clearly demonstrate that there is a close correlation between the levels of free cholesterol and free fatty acids in lymphoid cells from mice or guinea-pigs. Recently it has been reported that free cholesterol markedly increases the viscosity of lipid bilayer in the surface membrane of lymphocytes, resulting in the increase of rigidity in the surface membrane[5, 13, 14]. Thus it

[1] S. J. Singer and G. L. Nicolson, Science *175*, 720 (1972).
[2] A. D. Bangham, A. Rev. Biochem. *41*, 753 (1972).
[3] M. S. Bretscher, Science *181*, 622 (1973).
[4] S. J. Singer, Adv. Immun. *19*, 1 (1974).
[5] M. Shinitzky and M. Inbar, J. molec. Biol. *85*, 603 (1974).
[6] E. L. Gottfried, J. Lipid Res. *8*, 321 (1967).
[7] I. Vlodavsky and L. Sachs, Nature, Lond. *250*, 67 (1974).
[8] S. Kigoshi and M. Akiyama, Experientia *31*, 1225 (1975).
[9] S. Thunold, Acta path. microbiol. scand. *71*, 564 (1967).
[10] S. Kigoshi and R. Ito, Experientia *29*, 1408 (1973).
[11] J. N. Fain, Fedn. Proc. *29*, 1402 (1970).
[12] S. Kigoshi and R. Ito, Experientia *32*, 243 (1976).
[13] U. Cogan, M. Shinitzky, G. Weber and T. Nishida, Biochemistry *12*, 521 (1973).
[14] M. Inbar and M. Shinitzky, Proc. natn. Acad. Sci., USA *71*, 4229 (1974).

appears that, in lymphoid cells from mice or guinea-pigs, increase in the rigidity of surface membrane by free cholesterol is accompanied by increase of free fatty acids. Free fatty acids from various sources, including lymphocytes, have long been known to be highly cytotoxic to many mammalian cells[10,15–19]. TURNELL et al.[20] have also indicated that, in corticosteroid-sensitive lymphocytes, accumulation of free fatty acids is involved in corticosteroid-induced lymphocytolysis. Therefore it is conceivable that free cholesterol, which is believed to be present almost exclusively in the cell surface membrane[3], regulates the levels of cytotoxic free fatty acids in lymphoid cells by changing the rigidity of cell membrane. Concerning this, it is of special interesting that the facile exchange of free cholesterol occurs in vitro between the surface membrane of lymphocytes and the surrounding medium (liposomes) containing free cholesterol and lecithin[5], and unsaturated free fatty acids promote the membrane fluidity of lymphocytes; namely, they decrease the viscosity of lymphocyte membrane[21]. Free fatty acids in lymphocytes have been shown to consist of 65–70% unsaturated fatty acids[22].

In splenic lymphoid cells, no significant difference was found in the regression coefficient of fatty acid to cholesterol between mice and guinea-pigs, as described above. The regression coefficient of phospholipid to cholesterol for mouse splenic lymphoid cells was also similar to that for the corresponding cells from guinea-pigs (1.35 for mice and 1.20 for guinea-pigs, $p < 0.01$). In contrast, the regression coefficient of fatty acid to cholesterol as well as that of phospholipid to cholesterol in lymphoid cells from animals markedly differed from the original tissues. These differences in the regression coefficient among various tissues suggest that each of lymphoid cells from thymus, spleen, mesenteric lymph node or other lymph nodes shows a characteristic change in the composition of membrane lipid which is closely connected with the properties and functions of cell membrane.

[15] L. R. BENNET and F. E. CONNON, J. natn. Cancer Inst. *19*, 999 (1957).
[16] G. F. TOWNSEND, J. F. MORGAN and B. HAZLETT, Nature, Lond. *183*, 1270 (1959).
[17] S. M. MILCU, I. POTOP, R. HOLBAN-PETRESCU, V. BOERU, E. GHINEA and C. TASCA, Neoplasia *16*, 473 (1969).
[18] H. OKUDAIRA, T. KATAOKA, H. OKADA, K. FURUSE-IRIE, S. KAWACHI, S. NOJIMA and K. NISHIOKA, J. Biochem., Tokyo *68*, 379 (1970).
[19] A. KATO, K. ANDO, G. TAMURA and K. ARIMA, Cancer Res. *31*, 501 (1971).
[20] R. W. TURNELL, L. H. CLARKE and A. F. BOURTON, Cancer Res. *33*, 203 (1973).
[21] E. M. KOSOWER, N. S. KOSOWER, Z. FALTIN, A. DIVER, G. SALTOUN and A. FRENSDORF, Biochim. biophys. Acta *363*, 261 (1974).
[22] E. FERBER, G. G. DE PASQUALE and K. RESCH, Biochim. biophys. Acta *398*, 364 (1975).

Regulation of Enzymes of Ethanol Metabolism in Yeast (*Rhodotorula gracilis*)[1]

G. M. HANOZET, M. SIMONETTA, D. BARISIO and A. GUERRITORE

Department of General Physiology and Biochemistry, University of Milan, 50, Via Saldini, I–20133 Milano (Italy), 29 December 1975.

Summary. The three enzymes of ethanol metabolism alcohol dehydrogenase, aldehyde dehydrogenase and acetyl-CoA synthetase in the obligate aerobic yeast *Rhodotorula gracilis* are repressed by glucose and induced by C_2 metabolic fuels with a regulatory pattern indicating a correlation in the control mechanisms. To try an identification of the molecular signals involved in the transmission of the inducing stimulus, experiments were carried out by blocking with 2 m*M* pyrazole the ethanol $\rightarrow$ acetaldehyde metabolic step. Results indicate that ethanol is not specifically required as a molecular signal for induction.

An interesting aspect of control of cellular level of enzymes is the multiple response of a linked group of enzyme proteins to the same environmental change. In the present paper, results are reported concerning the response to the same inductive or repressive stimulus of three enzymes of ethanol metabolism in the yeast *Rhodotorula gracilis*. The three enzymes are alcohol dehydrogenase (EC 1.1.1.1), aldehyde dehydrogenase (EC 1.2.1.5) and acetyl-CoA synthetase (EC 6.2.1.1). Alcohol dehydrogenase in *Rhodotorula gracilis* – an obligate aerobic organism – is presumably not concerned with alcoholic fermentation, but with oxidative utilization of external alcohols or with internal metabolism of alcoholic compounds. Its level is under epigenetic control, being dependent on the following kinds of environmental effects: induction by ethanol, repression-inactivation by glucose, in vivo stabilization by Zn[2,3]. In addition to ethanol, a high inducing capacity is shown specifically by other C_2 compounds, especially by acetaldehyde[3]. In conditions of Zn deficiency, the action of glucose is not only that of a repressor: glucose also promotes an evident inactivation of enzyme protein in vivo[4], and addition of Zn prevents this inactivation. The nature of the agents that directly contribute to the transfer of the inductive or repressive stimulus is not yet understood. A large number of metabolic intermediates are among the suspected substances that can change their level and act as a regulatory signal. The results presented here show that not only alcohol dehydrogenase, but two other enzymes of the pathway ethanol $\rightarrow$ acetyl-CoA are under epigenetic control in the cell of *Rhodotorula gracilis*. The data indicate a correlated response and point to the existence of a common regulatory metabolic signal.

Materials and methods. The strain of *Rhodotorula gracilis*[4] was maintained on nutrient malt-agar slopes. Cells were grown on synthetic medium with the following composition: 15 m*M* $(NH_4)_2SO_4$; 8.6 m*M* NaCl; 5.7 m*M* K_2HPO_4; 4 m*M* $MgSO_4$; 2.3 m*M* $CaCl_2$; 0.018 m*M*

[1] This work was supported by a grant from the Italian Consiglio Nazionale delle Ricerche.
[2] A. GUERRITORE and G. M. HANOZET, Ital. J. Biochem. *22*, 244 (1973).
[3] G. M. HANOZET, M. SIMONETTA and A. GUERRITORE, Ital. J. Biochem. *23*, 56 (1974).
[4] A. GUERRITORE and G. M. HANOZET, Experientia *26*, 28 (1970).