Evidence of the Participation of Deoxycholate in Cancer Immunity

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The hypothesis of cancerostatic activity of deoxycholic acid, recently outlined, is supported by correlation of natural cancer resistance with the level of deoxycholic acid in animals and humans. Analyses of sera indicate lower levels of unconjugated deoxycholic acid in cancer patients, the mean values of other bile acids being normal. Application of this theory to statistics of cancer incidence reveals possibilities of new aspects.

In a previous paper [1], we have described a peculiar pH-dependence of the toxicity of deoxycholic acid (DCA): by lowering pH, a rapid onset of biological activity suddenly appears at pH 7.3. As a pH considerably lower than the physiological one is a common property of malignant tissues [2–4], even of micrometastases [5], we outlined a hypothesis that DCA might be a natural cancerostatic agent, active in mammals even at physiological concentrations. The present paper is a confrontation of this hypothesis with the present (very limited) knowledge of the extra-intestinal fate of bile acids (BA).

In an early work, a deficiency of BA was found in the blood of cancer patients [6]; the analytical method, and too high results in normals are, however, not acceptable at present. A relation between the primary malfunction of liver and the secondary cancer was extensively discussed in BLOND's book [7], but the possible role of BA in this mechanism was neglected. Therapeutic experiments with DCA in mice bearing Ehrlich's ascitic carcinoma were unsuccessful [8]; mice, however, are not suitable animals for these experiments (cf. below, *k*), and moreover, a continuous supply of DCA was not maintained. On the contrary, ARDENNE was able to get positive results in mice with high i.p. doses of DCA, followed by hyperthermy [9]. In the same paper, the author also demonstrated a pH-dependence of the toxicity of BA for EMAC-cells in vitro. He considers the pH-dependence to be a result of continuous dissociation; extrapolations of his curves limit to zero-toxicity: for DCA at pH 7.3, for chenodeoxycholic acid at pH 7.0.

DCA differs from other human BA by its ability to form inclusion compounds with many insoluble organic substances ("choleic acids"), polymers with a helical structure [10] at pH below 7.3, and by its known ability to increase the permeability of cellular membranes. In the background is a strong tendency to association; dimers are assumed even in very diluted aqueous solutions [11, 12]. At elevated pH, the association proceeds probably by Van der Waals forces, with oppositely oriented ionized carboxyl groups, and OH-groups directed outwards [13]. Interaction with protons may result in H-bond formation connected with a rearrangement to a different association form, more stable below pH 7.3, possibly with hiding of hydroxyl groups inside the aggregates. This process would need activation energy, and the transition state (not excluding total dissociation of dimers) should be especially reactive. As no second-order reaction is necessary, such a rearrangement can take place in a high yield by a slight change of the physicochemical conditions: ionic strength and, especially, $H^{\oplus}$-ion concentration.

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The "acid form" of DCA due to its increased lipophility, and steroid structure somewhat resembling glucocorticoids, should not be free of hormonal activity, especially in permanently acid tissues. Actually, the hypotensive and digitalis-like activity on the heart [14] have been known for many years [15]. Despite this, no attempt was ever made to incorporate DCA or other BA into the hormonal balance, that being accepted as a condition of the "non-cancerous state" of the human organism.

Notes on the Physiology of DCA

a) Primary BA in man, synthesized in the liver, are cholic acid and chenodeoxycholic acid. DCA is produced by intestinal dehydroxylating microflora. If the passage of bile through the intestine is interrupted by a bile fistula [16], or after application of antibiotics [17], DCA disappears from the bile.

b) Free DCA permeates through the intestinal wall by passive diffusion into portal blood; in the liver, it is conjugated, and as glyco- and (less) taurodeoxycholic acid stored in the gall-bladder. Another part reaches the systemic circulation via the lymphatic duct. Salts of conjugated BA, excreted with bile, are split by deconjugating intestinal bacteria [18]. For review cf. DIETSCHY [19].

c) In the sera of normals, the concentration of total DCA ranges from 0.05 to 0.5 μg/ml (mean 0.22), about one half being unconjugated. In hepatobiliary diseases, only the primary BA in conjugated form are greatly increased (by more than 2 orders) [20–22].

d) On the skin, even of patients with hepatobiliary disease, the unconjugated DCA predominates [23]. It indicates the possibility that DCA is preferred to other BA in the transport through tissues.

e) The passage of BA from the blood into tissues seems to be slow, taking several days [23–25]. Hence, an acute onset of biological activity of DCA in muscles that are transitorily acidified [26] is hardly to be expected, but slightly increased effects seem probable, especially in the heart.

f) Glycolysing tissues, the brain and the retina, are separated from the blood circulation by the brain-blood and eye-blood barriers; the passage of DCA is not probable (cf. *g*). A growing tumor may destroy this barrier and permit the entry of DCA [27].

g) Unconjugated DCA is not present in the sera of newborn children. It seems possible that unconjugated DCA does not pass the placenta. Children under 1 year mostly have no DCA, after 1 year of age the serum BA pattern becomes equal to that of adults due to the establishment of normal intestinal microflora [28].

h) In the serum, more than 90% of BA are bound to proteins [20]; high affinity is assumed to ε-aminogroups of lysine in albumins [29]. (Note: the same type of binding is assumed for acid dyestuffs used for detection of the albumin deficit connected with cancer [30].)

i) Anti-A-helixagglutinin (an antibody-like substance from *Helix pomatia*) was shown to have an enormous capacity to bind DCA [31]. Should the human anti-A-agglutinins have a similar affinity to DCA, and, speculatively, anti-B-agglutinins miss it, then people of blood group B could be predisposed to a higher accumulation of DCA in their tissues.

j) The rabbit synthesizes mainly cholic acid in the liver, but glycodeoxycholic acid is practically the only BA in the bile of adults. A high proportion in the intestine is deconjugated, and the total concentration in the blood [32] is 3 – 30 μg/ml (compare with *c*). Besides traces of allodeoxycholic acid, neither BA different from human BA, nor appreciable amounts of chenodeoxycholic acid were found.

k) Rats and mice have cholic, muricholic, hyodeoxycholic, chenodeoxycholic, and a small proportion (5% in the rat) of (total) DCA in the portal

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blood [19]; in the bile ursodeoxycholic acid was also found. Concentration in peripheral blood and degree of deconjugation are unknown.. In the liver, rehydroxylation of DCA to cholic acid proceeds [16], and no DCA has been noted for the bile [33]. In a recent work, however, 17% of (total) DCA in the bile of Wistar rats was found [34]; possibly great variations between different strains exist. Conjugation is of 95% with taurine; adrenalectomy greatly increases the conjugation with glycine [35]. Domestic fowl, like other birds, have no DCA in the bile [33].

l) The high content of DCA in rabbits, and low proportion of dihydroxycholanic acids in rats have been put into relation to the proneness of rabbits, and resistance of rats to develop atherosclerosis on cholesterol feeding. In humans, a significant direct correlation between the degree of atherosclerosis and the ratio of dihydroxycholanic acids (DCA + chenodeoxycholic acid) to cholic acid in the gallbladder was found [36].

m) BA, and especially the most effective DCA, are considered to be regulatory agents in oxidative phosphorylation. ATPase activity and various synthetic and transport systems (including that of glucose) [14, 37].

Cancer in Animals and the Level of DCA

The susceptibility of laboratory mice, rats, and hens to spontaneous, carcinogen- and virus-induced tumors is well known; spontaneous regression [38] is less than 1 per cent. The deficiency of DCA in these animals has been discussed above (*k*); the assumed variations in DCA levels between strains may contribute to variations in cancer susceptibility.

In rabbits, with a high level of DCA (*j*), spontaneous tumors are rare, the action of carcinogens is slower and effective in a far lower percentage than in mice and rats [28, 39]. Cirrhosis of the liver preceeds, rather then follows, the development of carcinomas after diethylnitrosamine feeding [7, 40], i.e. the natural metabolism of BA is destroyed first. The tar cancerization of the skin as well as the preference of areas treated with oils (even non-carcinogenic) [41] after intravenous virus application might be enhanced by the elimination of DCA, or by the formation of choleic acids. The Shope papilloma is benign with high regression [38]. The Shope fibroma heals spontaneously in adult rabbits [42]. Regression of the Brown-Pearce tumor is 93% after intra-, 80% after subcutannous, 67% after intramuscular inoculation [38]. Implanation of different tumors into the eye of the rabbit is, generally, successful, but regression takes place again, after some degree of growth has been reached [38]; this is compatible with *f*.

Limitations of Experiments in Animals

The most suitable animals for cancer induction, i.e. mice, rats and hens, do not have their BA-metabolism adapted for accumulation of DCA (*k*). Hence, even i.p. administration is doubtful, if physiological doses are considered that do not exceed the metabolic capacity of the liver.

Further, different species have different BA that might, in an unknown degree, compete with DCA — or substitute it, or potentiate its biological activity. Hence, results obtained in animals would be hardly applicable to men.

As metabolic pathways of DCA in animals lacking DCA are not yet known, practically only the rabbit, with a high natural level of DCA (*j*) comes into consideration among usual laboratory animals. The rabbit deprived of its natural DCA should lose its resistance against cancer, according to the theory proposed. This might be possible by bile fistula, or germ-free breeding, or (partially only [34]?) by massive feeding with antibiotics. However, use can be also taken of the very probable absence of uncon-

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jugated DCA in newborn rabbits, as is the case with newborn children (*g*).

Immunity, Antibodies, and DCA in Rabbits

The growth and regression of Shope fibroma in adult and newborn rabbits has been studied by several authors, recently by ALLISON [42]. After inoculation of adults with the virus, "... the tumors continued to grow large until about the 8th or 9th days. At about this time depression and hemorrhagic necrosis began to appear in the tumors... Necrosis spread through the tumors which sloughed off by about the 16th day and left scabbed ulcers that rapidly healed and contracted." In contrary, newborn rabbits inoculated in the 2 – 5th days after birth "... all had tumors that grew progressively until they were nearly as large as the baby rabbits themselves. Satellite tumors, secondary tumors in the lungs, liver and spleen were common. No sign of regression was observed, even in those surviving for as long as 24 days. All died. Fibromas in newborn rabbits were from the beginning more cellular, with less exudate and little inflammatory reaction. The tumors looked sarcoma-like and sometimes invaded muscles." Implantation of lymphatic node cells (from immune rabbits) had no effect. Formation of antibodies started in both groups simultaneously, the titre was comparable. The author concludes: "The humoral antibody response in newborn rabbits is not greatly different from that in adult rabbits; the cellular immune response is however slight."

Resulting from the previous experiment, some questions arise that could become working hypotheses for further investigation: Are antibodies in the absence of DCA ineffective against tumors? Is DCA, generally, essential for an effective cellular immune response? May some antibodies be specific "BA binding proteins" (in analogy to transcortin or the sex steroid binding protein)? Affinity of some surface-active salts to different antibodies has been demonstrated [43]; cf. also *i* *.

Bile Acids in Cancerous Sera

As announced in our first paper [1], we performed some analyses of serum BA in order to verify whether malignant growth is associated with a lower level of DCA. The analytical method included separation using anion exchange and gas-liquid chromatography [20]. For calibration, a pooled sample of 16 normals was applied, the total BA content of which was put equal to the known mean [20, 22]. Cholic acid was not evaluated because of its low response in the given chromatographic system; chenodeoxycholic acid (CDCA) was the only representative of primary BA. The concentrations of total BA ($CDCA_t$, DCA_t) were evaluated from analyses including hydrolysis, for those of unconjugated BA ($CDCA_u$, DCA_u) the hydrolytic step was omitted; some possible losses in hydrolysis were neglected.

Mean values of the analyses of 8 cancer patients (3 endometrial, 2 cervical, and 3 prim. lung carcinomas, with no hepatobiliary disorder proved) are summarized in Table I. It is seen that the level of DCA_t is not changed, at least in developed cancer; it means that an insufficiency of the intestinal dehydroxylation system (*a*) will be, generally, of minor importance. The decrease in the level of DCA_u might be due to low activity of the deconjugating bacteria (*b*), but a bad intestinal absorption of DCA_u seems more probable. A "malabsorption syndrome" is common in cancerous organisms [44].

	$CDCA_t$	$CDCA_u$	DCA_t	DCA_u
Normal (pooled sample)	0.26	0.07	0.22	0.11
Cancer mean	0.27	0.06	0.22	0.05
range	0.10 – 0.54	0.02 – 0.13	0.07 – 0.56	0.00 – 0.11

Table I. Bile acids in normal and cancerous sera (μg/ml).

These preliminary results – of few sera collected at random – indicate the expected parallelism between cancer and deficiency of unconjugated DCA in men. However, as its level in the blood may be more variable than the level in tissues (cf. *e*), the determination of the latter would be more adequate (not described yet with the exception of animal liver and gut [34]), or the stability of the former should be established first (dependence on ingested food is probable). Determination in the urine is not

* The anticipated promotion of immunity by DCA will be checked also in some other viral infections.

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yet possible at normal levels [45]; an attempt will be made to elaborate a sensitive method.

For verification of the theory, patients should be selected with great care. If analyses are made during stationary phase of the malignant process, the DCA-level may be normal. On the other hand, in a single pooled sample of 4 cases of laesio cervicis uteri (with precanc. epithelium), we found an almost complete absence of all dihydroxycholanic acids.

Indirect Evidence of Cancer: DCA Antiparallelism in Humans

The degree of atherosclerosis has been put into direct relation with the level of dihydroxycholanic acids (*l*), and the degree of (calcified) atherosclerosis has been found to be in inverse correlation with cancer (in a great number of post mortem examinations [46]). Connecting these two separate observations, it follows that a parallelism between deficiency in (total) dihydroxycholanic acids and cancer has been demonstrated. Respecting *d* and *l* and considering DCA_u to be, on the average, proportional to DCA_t, it results that a parallelism of DCA_u-level and cancer resistance is highly probable.

Myomas are mostly benign, and rare in active muscles (especially cardomyoma with a tendency to regression). This is compatible with the assumption of a somewhat higher activity of DCA in muscles (*e*) and in the heart [14].

Hepatobiliary diseases were sometimes considered as a precancerous state, and many references are summarized in BLOND's book [7]. Should DCA be involved in this mechanism, it could be assumed that DCA in tissues is competitively inhibited by the large excess of primary BA (*c*), or DCA production and transport into blood is impaired due to low secretion of bile into the intestine; diets depressing bile production might be a further contribution. Statistical evaluation seems desirable.

Antibiotics, chemically preserved food, and other peroral antiseptics may depress the intestinal production of secondary BA as well as deconjugation (*a, b*). This mechanism might mediate between abuse of germicides and the steady increase in cancer incidence. On the other hand – does not a closer contact with the soil (containing BA-transforming bacteria [47]) contribute to lower cancer incidence in the country?

In cancer patients, the blood group A prevails; correlation is poor for cancer of sex-hormone target organs, striking for tumors of salivary glands, significant for cancer of the stomach and some other neoplasms [48]. Using the hypothesis of a lower accumulation of DCA in tissues lacking anti-A-agglutinin (*i*) and accepting a sufficient supply of salivary glands with agglutinins, this result is compatible with the assumed participation of anti-A-agglutinin in DCA distribution.

Persons with allergies, espec. with bronchial asthma, are commonly considered as somewhat more cancer resistant [49, 50], even if this relation was disputed [51]. We did not (preliminarily) detect any substantially higher DCA-level in the blood of asthmatics, but it is not excluded that the content in tissues might be higher; in asthmatics, a predomination of blood group B was found [48], although disputable [52]. Besides, high cellular antibody level may be suitable terrain for an accumulation of DCA (cf. above). The well-known remission of allergic symptoms in hepatobiliary diseases, starvation and after antibiotics, is also compatible with the assumption of an effective natural supply of tissues, perhaps predisposed, with DCA.

In children after 1 year of age, DCA level becomes equal to that of adults (*g*), but corticoids and sex hormones not until adolescence. Within this interval, cancer is rare, and allergies are frequent.

Some groups of cancer patients have been shown to have an anomal corticoid metabolism many years

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before the diagnosis of cancer [53, 54], and to have symptoms of adrenal cortex hyperfunction [55]. Glucocorticoids are known to play an important role in malignant growth, and in suppressing the immune response (generally, including cancer induction and allergic reactions). The serum concentration of cortisol (cca $0.05-0.18\,\mu g/ml$) is very near to the mean of DCA_u. Elucidation of the role of DCA in the human organism would not be complete without consideration of a possible competition of these abundant steroids, with known effects on membrane permeability and cellular metabolism.

Prospects in Cancer Prevention or Treatment

As demonstrated, a number of facts support the hypothesis of a positive role of DCA in natural cancer immunity. After elaboration of practical analytical methods, we hope that extensive studies in human population may result in finding a physiological (or permanently tolerable) level of DCA, effective in the presence of other BA and neutral steroids. A reasonable first approach could be studies of individuals with spontaneous regression of tumors, and of people with special symptoms appearing, according to statistics, more resistant. Should this causal relation be reliably verified, safe methods for attainment of this "protective" level should be elaborated, preferring, wherever possible, dietary regulations. (Note: Cytostatics could impair the terrain for DCA-activity; in irradiated and highly degenerated human tumors, the pH was found to be only negligibly lower [56].)

Parallely, studies on the biological activity of chemically altered BA should not be omitted, respecting first transformations that take place to a minor extent in the intestine (in allergies, dysmicrobia is frequent), or are performable by saprophytic microflora. It may be that a clue to new aspects of hormonal regulations will be revealed in steroid acids.

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