Institute of Pathological Anatomy (Director: Prof. Gy. Romhányi) Medical University, Pécs

THE HISTOCHEMISTRY OF UNSATURATED LIPIDS WITH REFERENCE TO THE ADRENALS

Anna Horváth and K. Jobst

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The studies followed for several decades by the Kendall and Reichstein schools opened a new stage of steroid research in 1953 when Reichstein, Simpson and Wettstein isolated aldosterone as a further biologically active compound of the adrenal lipid complex [35]. The favourable therapeutical results called attention to the significance of the adrenal cortical hormones, although their action mechanism is even now far from clear. The efforts to synthetize these hormones in the laboratory were largely successful but very little had been known of their biosynthesis. An insight, slightly closer than the one based on mere assumption, into their process of anabolism was obtained only in recent years by the isotope technique. Bloch, Cornforth, Clayton [5, 6, 7] and others, using C^{14} labelled acetate, studied the synthesis of cholesterol *in vivo*, revealing the process of conversion from the saturated acetate molecule *via* mevalonic acid and unsaturated squalene to cyclic molecule. Werbin and Leroy demonstrated by the same means the transformation of cholesterol into biologically active steroids [33, 34]. According to their findings, the biosynthetical process leads from saturated open-chain molecules by closure of the chain *via* unsaturated intermediates to hydroaromatic compounds of the cyclopentano-perhydrophenanthrene type which forms the biologically most important group of the sexual and adrenocortical hormones.

Soon after the biological function of the adrenal steroids had been elucidated, morphological and histochemical inquiries were started to demonstrate and localize the active components *in situ*. However, owing to the close structural similarity of the components, the lipid-steroid extracts could be fractionated and identified even *in vitro* by rather cumbersome methods. Since the same experimental conditions cannot be ensured in living tissues, it is still too early to speak of a histochemical lipid analysis. Inferences as to the distribution of lipids are drawn from the localization of neutral fats, established by Nile-blue staining and the plasmal and Schultz reactions [10, 32]. Considering the major role played by the biologically inactive compounds in the positive reaction of neutral fats, until more specific histochemical steroid reactions will be worked out to modify our present knowledge of steroid byosyn-

thesis and intermediary lipid metabolism, the functional state of the adrenal is more appropriately characterized by its unsaturated lipids than by the quantitative distribution of the total lipids.

On the basis of these considerations, we have studied the histochemistry of unsaturated lipids, with special reference to the adrenal. In the present paper we shall report on our own method, with emphasis on the mode of fixation.

Two qualities of the ethylene bond suggest themselves as a means for the histochemical determination of unsaturated lipids, viz. a) the oxidative cleavability [2, 20] and b) the capacity to react with haloids [38]. In the former the reduction of the resulting aldehyde groups, in the latter the appearance of metallic silver in the course of the secondary reaction, indicates the site of the double bond.

Oxidative splitting forms the basis of PEARSE's performic acid [29], LILLIE's peracetic acid [23], BELT and HAYES' ultraviolet Schiff reactions [4] and of HOLCZINGER's auto-oxidation method [15]. The reaction of the double bond forms the basis of the methods described by LILLIE [23], BARROLIER and SUCHOWSKY [3], NORTON and KOREY [28], MUKHERJI [26], and the one known as the osmium method [37]. Both reaction types are characteristic mainly of the aliphatic ethylene bond, but the oxidative process has a further effect, causing the C_{20}—C_{21} corticosteroid atoms to form reducing groups [18]. To avoid this, we gave preference to the halogen reaction for the determination of unsaturated lipids.

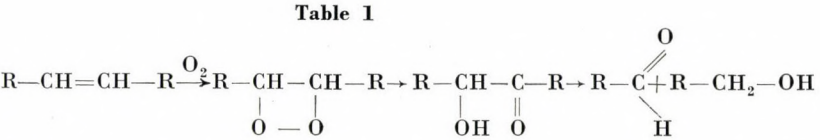
Experimental

Both the oxidative and the halogen methods work quantitatively under strictly controlled conditions. It is therefore not surprising that different histochemical methods should have given equivocal results with the same material. These we have found to depend on (i) the mode of fixation ; (ii) the activity of reagents; (iii) the reaction time; (iv) the fat solubility of reagents (heterogeneous phase): (v) the way to indicate the reaction product.

(i) *Fixation.* — The adrenal fixed in 10 per cent formalin for more than 72 hours showed gradual diminution of peracetic acid Schiff positivity, with the simultaneous appearance of the Schiff pseudoplasmal reaction [8] and transitory PAS positivity [40] (see Table 1).

After formalin fixation there was no notable lipid dissolution, but some destruction of the submicroscopic structure of the lipid-protein complex occurred, accompanied by auto-oxidation of the double bonds [14, 17, 29], a phenomenon described by GOMORI [13] and

Table 1



Peracetic acid Schiff	+	—	—
Halogen additin	+	—	—
PAS	—(+)	+	+
Leukofuchsin (pseudoplasmal)	—	(+)	+
Odour	—	+	+

LILLIE [23]. This property of the unsaturated lipids forms the basis of DEANE's [8] and HOLCZINGER's [15] method. The fat peroxides and secondary aldehydes (pseudoplasmal) are formed from the double bonds under the effect of the photochemical reaction of the oxygen absorbed by the fixative or by the catalytic effect of the dissolved trace metals (copper, iron) [9, 17]. When the formalin-fixed organs rich in fat are exposed to light, a distinct rancid smell betrays the process of auto-oxidation which is enhanced in the presence of oxidants, usually carotenoid pigments accompanying the lipids [9]. It was attempted to inhibit the chain reaction with different anti-oxidants, but neither the use of certain reducing substances such as phenol or quinone derivatives or gallic-tannic or ascorbic acid, nor the introduction of catalyzing trace metal complexes (EDTA-Na_2) has solved the problem satisfactorily [9, 17].

Since the coloured oxidation products of polyoxiphenols (pyrogallol, hydroquinone) are dyeing the tissues and metal catalysis occurs rarely with photo-oxidation, we applied as an anti-oxidant sodium formaldehyde sulphoxylate ($HO-CH_2 \cdot SO_2Na$), generally known by the commercial name of rongalit or redit C. As its hydroxyl enters in condensation reaction with the amino-groups [16], the compound has a protein fixing effect besides the reducing property of its sulphoxyl radical. It was found superior to formaldehyde for the preservation of unsaturated lipids [11]. Good results were obtained with a 3 per cent solution of rongalit in 5 to 10 per cent formaldehyde (Figs. 1 and 2), since the lipochromes (carotenoids) in the preserved specimens retained their original yellow colour as a sign that the fixative inhibited the auto-oxidation of the otherwise easily oxidizing molecule. Ethylene reactions of one type, carried on with the same material for several days or even weeks, yielded invariably and in a well reproducible manner identical results, while every other method yielded results greatly different in intensity and distribution. This experience has led us to study the additional halogen reaction of the ethylene bond.

(ii) The *activity of halogens.* — Considering that preparations, purified *in vitro* and treated with iodine or Lugol's solution, do not give a yield higher than 50 to 60 per cent of the theoretical iodine number, HÜBL, WIJS and HANAUS were using either catalysts or the much more reactive interhaloid derivatives of iodine [38]. In our own choice of bromine chloride as the most suitable type of haloid bromine derivative whose qualities had been thoroughly revealed in experiments *in vitro*, we were led by the consideration that it is readily entering an additive reaction with double bonds and its reaction rate surpasses that of bromine and especially of chlorine [25].

(iii) *Reaction time.* — The fact, revealed *in vitro* and confirmed by our histochemical findings, that bromine chloride takes 6 hours to produce a state of maximum saturation, in contrast with the 24 hour needed by elementary bromine [25], has induced us to regard 6 hours as an optimum length of time for bromine chloride to be bound quantitatively to unsaturated and therefore more slowly responding lipids. The usual shortness of the time factor calls for caution in interpreting the results of the additive methods as described in the literature.

(iv) *Solubility of reagents — the heterogeneous phase.* — The most critical stage for demonstrating lipids histochemically occurs during the heterogeneous phase. The efficiency of the reagents in aqueous media depends mainly on their lipid solubility. According to ADAMS [1], the ethylene bond of the polar lipids is able to react with iodine only, whereas with bromine every double bond is reacting. PEARSE used a carbon tetrachloride solution of bromine to ensure optimum conditions, namely the homogeneous phase, but the method involved a loss of fat owing to the solvent nature of carbon tetrachloride. The lately adopted practice to use bromine vapour [26] results in shrinkage and a rubber-like appearance of the sections. This greatly disturbs the interpretation of fine details, quite apart from the fact that the readiness of bromine to react with 1—3 glycol bonds (glycogens) makes the selectivity questionable [26, 28]. Bromine chloride, on the other hand, does not react with glycol, is less potent as an oxidant and has a fat solubility not worse than that of pure bromine, so that a positive reaction is mainly due to the ethylene bonds. The reagent is easy to prepare according to SCHULEK and BURGER [3], as in the presence of hydrochloric acid, the bromate and bromide ions are reacting with the quantitative formation of bromine chloride, $BrO_3^- + 2Br^- + 3Cl^- + 6H^+ = 3BrCl + 3H_20$

The reagent can be stored in a filled dark bottle with glass stopper.

(v) *The final reaction point.* — Silver salts are mostly used to indicate the halogens reacting additively with the double bonds. The sites of the latter are marked by the appearance of black colour which originates from metallic silver given off by the haloid. However, bromine is not split off from di-bromine derivatives by an aqueous solution of silver nitrate. Of the alpha-beta interhaloid derivatives it is mostly the more electro-negative component that can be transformed into halogenoid ions after alkaline mineralization [31] (see Table 2). According to the basic equation chlorine as the more electro-negative ion is split off quantitatively and is free to react with the silver ions. The two prerequisites, viz. the possibilities of splitting off and

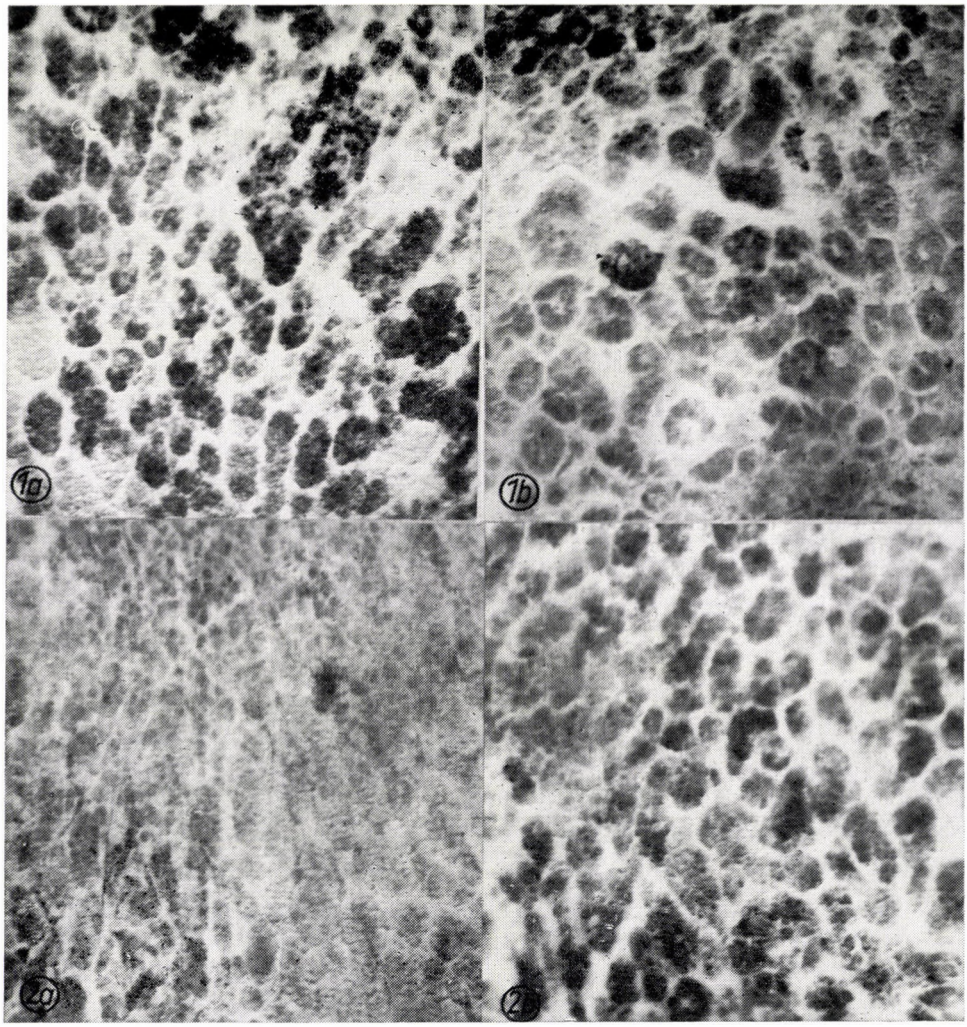
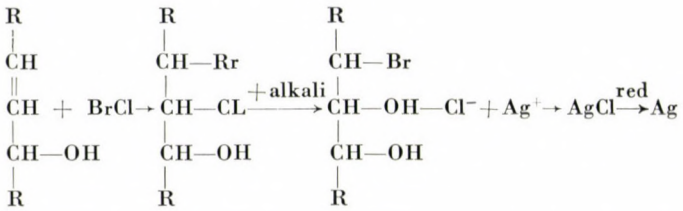


Fig. 1. Frozen section of human adrenal fixed for one month *a)* in 10% formalin; *b)* in a mixture of formalin and rongalit. Schiff (pseudoplasmal) reaction. The reaction is more intensive in the formalin-fixed section

Fig. 2. Frozen section of human adrenal fixed for one month in *a)* 10% formalin; *b)* in a mixture of formalin and rongalit. Peracetic acid Schiff reaction

The amount of unsaturated lipids in the formalin-fixed material is considerably less. The Schiff-positive components arising under the effect of auto-oxidation have been eliminated prior to the reaction

Table 2



silver haloid formation, are ensured in the silver diamine complex $Ag(NH_3)_2OH$. The metallic silver can be reduced by development from the resulting insoluble precipitate. At the same time, as the acid reaction of the aqueous silver nitrate solution does not ensure a full splitting off of the halogenoid ion, the quantitative nature of the reaction is somewhat questionable.

Our own method of indicating the double bonds of unsaturated lipids, evolved under due consideration of the above points, consists of the following phases.

(i) Fixing in a solution of 3 per cent rongalit in 5 to 10 per cent formaldehyde.
(ii) Repeated washing of the frozen sections in distilled water.
(iii) Six hours' halogen treatment in n/10 ClBr prepared by the addition of 20 mg n/10 $KBrO_3$ to 10 ml of distilled water and 10 ml of 20 per cent HCl, and two hours' treatment in n/10 Lugol's solution.
(iv) 3 per cent sodium thiosulphate, twice 10 minutes each.
(v) Washing in distilled water.
(vi) Fontana's silver solution $Ag(NH_3)_2OH$, 20 minutes.
(vii) Washing in distilled water, twice 10 minutes each.
(viii) Rinsing in n/10 nitric acid, 5 minutes.
(ix) Washing in distilled water, twice.
(x) Methol hydroquinone developer, 20 minutes.
(xi) 3 per cent sodium thiosulphate, 5 minutes.
(xii) Rinsing in water, covering in glycerol or gum-arabic.

Results

We have studied the distribution of unsaturated lipids in adrenals, corpus luteum and retina. The fact that preparations fixed for several months showed the same reactions as fresh specimens, seemed to prove that the mixture of formalin and rongalit used by us was able to prevent the auto-oxidation of the ethylene bond and thus to allow the demonstration of peroxides originating from fatty acids *in vivo* [19]. Once the development of oxidation artefacts in the preserved specimens has been successfully inhibited, it was possible to conclude from a positive reaction to an accumulation of unsaturated compounds of the intermediary lipid metabolism. The simultaneous bromine chloride and iodine reactions furnished some information regarding not only the total amount of unsaturated compounds, but also the distribution of unsaturated polar lipids. According to our experience, more intensive and more widely extended reactions are presented by the preparations treated with bromine chloride than by those treated with iodine (Fig. 3). However, the role of steroid double bonds in the positive reactions is still to be clarified. Such studies are now under progress.

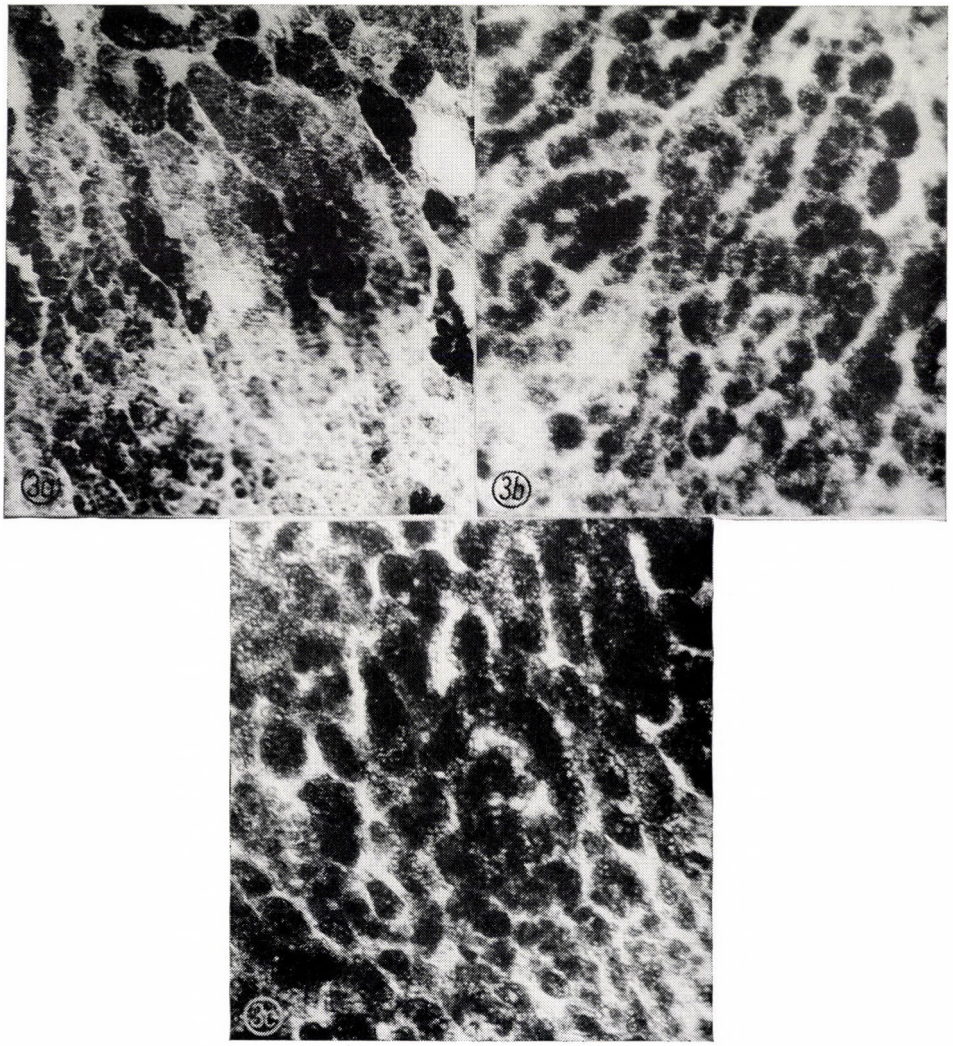


Fig. 3. Unsaturated lipids in human adrenal fixed for three weeks in *a)* formalin. Iodine staining (Lugol's solution) — *b)* in a mixture of formalin and rongalit. Iodine staining (Lugol's solution) — *c)* in a mixture of formalin and rongalit. Bromine chloride treatment and silver impregnation. Unsaturated compounds more in (*b*) than in (*a*) — most intensive reaction in (*c*)

Until some way has been found for the unsaturated intermediaries of lipid metabolism to be isolated and demonstrated *in situ*, inferences concerning adrenal activity and lipid synthesis can only be drawn from the total amount of unsaturated compounds [26, 27, 36]. However, some recent biochemical findings, compared with long established morphological facts, seem to bear out the following considerations.

There is evidence that the different lipid-storing organs almost invariably contain certain carotenoid yellow dyes. Equally consistent in the adrenals of aged subjects at the line of demarcation between cortex and medulla is the occurrence of a zone of yellowish-brown, resin-like material weakly soluble in fat solvents and considered by GOMORI [13], LILLIE [23] and WOLMAN [41] to consist of polymerized oxidation products of unsaturated lipids. A lipochromatic zone of similar appearance is to be found neither in juveniles nor in the hormonally inactive cortical adenomas of adults.

Conjugated polyene carotenoids polymerize in air to a resin-like insoluble product, the physical feature of which show a close relationship to those of adrenal lipochromes [29]. This can be brought into correlation with our recent knowledge regarding lipid biosynthesis, namely that squalene [21], steroids [22, 43] and carotenoids built up from isoprene all have the acetate molecule as their common basis [24]. This is transformed through β-hydroxy-β-methylglutaric acid into β-hydroxy-β-methylglutaric aldehyde (mevaldic) acid. Fromthis partly cholesterol and steroids are synthesized through mevalonic acid and squalene, partly also β-methylglutaconic-aldehyde acid, and thus by polymerization isoprenoids and then carotenoids [42]. There is evidence of the acetate and mevalonic acid molecule to be built in enzymatically into both cholesterol and β-carotene in lower organisms [12].

Since steroids and carotenoids occur together in the adrenal cortex, there might be morphological signs of a shift towards the synthesis of one or the other form the intermediary products of lipid metabolism. In active oxygen consuming processes the corticosteroids with a primary metabolic control effect are probably synthesized so vigorously as to oppress the carotenoid series. Increased oxygenization is apt to lead to an oxidative polymerization of the sensitive carotenoids, giving rise in the adrenal cortex to a zone of insoluble lipochromes which is interpretable as a morphological index for the described hyperactive functional state of the adrenal cortex.

Summary

The histochemical analysis of unsaturated lipids is usually disturbed by auto-oxidative reducing artefacts, developing during fixation. Their development has been successfully inhibited by the use of a mixture of formalin and rongalit, acting at once as fixative and reducing agent. Under consideration of the activity, solubility and demonstration of halogens and the length of time they take in entering additive reactions, a new method has been evolved for the histochemical demonstration of unsaturated ethylene-type bonds on the basis

of an additive reaction with bromine chloride. Morphological and recent biochemical findings have been compared to stress the significance of unsaturated lipids in both steroid and carotenoid synthesis, and in the interpretation of the functional state of the adrenals.

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О ГИСТОХИМИИ НЕНАСЫЩЕННЫХ ЛИПОИДОВ, С ОСОБЫМ УЧЕТОМ НАДПОЧЕЧНИКОВ

А. ХОРВАТ и К. ЙОБСТ

Авторы обращают внимание на самоокислительные восстановительные артефакты, возникающие при гистохимическом анализе ненасыщенных липоидов в связи с фиксацией. Для предотвращения этого явления они успешно применяли фиксирующий раствор Ронгалитформалин, обладающий восстановительным и одновременно фиксирующим свойствами. После исследования присоединяющей активности, времени и растворимости галогенов, и способа выявления галогенного атома, авторы разработали методику, основанную на присоединение бромхлора, для гистохимического демонстрирования ненасыщенных связей этиленового типа. Сравнивая морфологические и новейшие гистохимические данные они указывают на значение ненасыщенных липоидов в синтезе стероидов и каротиноидов, и в исследовании функционального состояния надпочечников.

ÜBER DIE HISTOCHEMIE DER UNGESÄTTIGTEN LIPOIDE, UNTER BESONDERER BERÜCKSICHTIGUNG DER NEBENNIERE

ANNA KOVÁCS und K. JOBST

Verfasser lenken die Aufmerksamkeit auf die bei der histochemischen Analyse der ungesättigten Lipoide anläßlich der Fixierung entstehenden reduzierenden Autooxydationskunstprodukte. Um deren Zustandekommen zu verhindern, benutzten sie als Fixiermittel Rongalit-Formalin, das auch zugleich reduziert. Unter Berücksichtigung der Additionsaktivität, Additionsdauer und Löslichkeit der Halogene sowie der Indikationsart des Halogenatoms arbeiteten sie eine auf Bromchlor-Addition beruhende Methode zur histochemischen Demonstration der äthylenartigen ungesättigten Bindungen aus. Unter Zusammenfassung der morphologischen und neueren biochemischen Ergebnisse wird auf die Bedeutung der ungesättigten Lipoide in der Steroid- und Carotinoidsynthese und bei der Beurteilung des funktionellen Nebennierenzustandes hingewiesen.

Dr. Anna HORVÁTH: Pécs, Orvostudományi Egyetem Kórbonctani Intézet, Hungary

Dr. Kázmér JOBST: Pécs, Orvostudományi Egyetem Kórbonctani Intézet, Hungary