Tabelle

	Isoxanthopterin und Derivate				Gelbes Pterin				Photolabiles Pterin			
	Gelbtiere	Schwarztiere	n	P	Gelbtiere	Schwarztiere	n	P	Gelbtiere	Schwarztiere	n	P
Rumpf	626	468	39	0,0002	340	247	39	0,0002	290	210	39	0,0002
Schwanz	339	332	39	>0,05	303	298	39	>0,05	211	192	39	>0,05
Auge	—*	—*	—*	—*	213	156	9	0,0002	40	22	9	0,0002

* Substanzmenge für eine vergleichende Bestimmung zu klein

Es handelt sich dabei um Isoxanthopterin, ein dem Isoxanthopterin nahestehendes Pterin, 2-Amino-4-oxypterin, ein gelbes sowie ein stark photolabiles Pterin (vgl. ZIEGLER-GÜNDER[6]). Letzteres dürfte wie bei Drosophila eine Tetrahydro-Verbindung sein, jedoch ist die Konstitution sowohl des gelben als auch des photolabilen Pterins noch nicht geklärt.

Es erhob sich nun die Frage, ob der morphologische Farbwechsel des Feuersalamanders von einer quantitativen Verschiebung in der Menge der Pterine begleitet ist.

Dazu wurden je 17 junge Larven dem Uterus eines Muttertieres entnommen und wechselweise in zwei Glasbecken gesetzt, die mit Plaka-Farbe allseitig schwarz bzw. gelb gestrichen und mit Zaponlack überzogen waren. Nach 6 Wochen (Wassertemperatur 14–16° C, Fütterung mit lebenden Tubifex) standen die Tiere kurz vor der Metamorphose.

Die auf gelbem Untergrund gezogenen Tiere (=Gelbtiere) zeigten wesentlich grössere gelbe Areale als die Schwarztiere: die Melaninbezirke zwischen den einzelnen gelben Flecken waren im Vergleich zu den Schwarztieren teilweise verschwunden und mit gelbem Pigment erfüllt, so dass bei den meisten Tieren neben zwei durchgehenden gelben Längsstreifen ausgedehnte Gelbareale an den Flanken entstanden waren.

Zur quantitativen Bestimmung der Pterine wurde die Haut mit der anhängenden Muskulatur abpräpariert, in zwei Teile geteilt (1. Rumpf: zwischen Vorderbeinen und Hinterbeinen; 2. Schwanzteil), sowie die Augen ausgenommen und alles im Dunkeln bei 60° C getrocknet. Die fein zerriebenen Proben wurden mehrmals mit $n/10$ HCl bei 50° C im Dunkeln 5–6 h lang extrahiert und die vereinigten Extrakte im Vakuum über KOH zur Trockne eingedampft. Nach Auflösen in einer konstanten Menge $n/10$ NH_4OH und Chromatographie gleichgrosser Proben mit Butanol/Eisessig/Wasser (4:1:5) ergaben sich 3 Pterinzonen: eine violettblau fluoreszierende (vorwiegend Isoxanthopterin und ihm nahestehende Pterinezone), das gelbgefärbte und gelb fluoreszierende sowie das stark photolabile Pterin[7].

Die quantitative Ausmessung erfolgte mit einem Fluorometer zur Auswertung von Papierchromatogrammen (vgl. KÜHN[8]) und ergab folgende Resultate (Tabelle).

Wir sehen, dass in der *Rumpfregion* bei den Gelbtieren sämtliche Pterine – nicht nur das als gelblicher Farbstoff sichtbare – um etwa 35% zunehmen. Die Differenzen sind gut gesichert.

Auch das gelbe Pterin im *retinalen Pigmentepithel* steigt um ungefähr den gleichen Betrag (Differenz gut gesichert), während das photolabile sogar um 82% gegenüber den Schwarztieren vermehrt ist.

Der «morphologische» Farbwechsel ist beim Feuersalamander somit von einer quantitativen Verschiebung in der Pterinmenge begleitet. Weitere Untersuchungen müssten zeigen, ob auch andere Pigmente – zum Beispiel das in der Epidermis der gelben Areale gelegene Carotinoid – gleichsinnige Veränderungen aufweisen.

Bemerkenswert ist die starke Zunahme des *photolabilen* Pterins in retinalen Pigmentepithel der Gelbtiere im Gegensatz zur Haut, wo das Verhältnis gelbes Pterin/photolabiles Pterin konstant bleibt. Die relativ stärkere Zunahme des photolabilen Pterins kann einerseits durch eine stetig höhere Zuwachsrate, andererseits durch ein rascheres Einsetzen der Synthese im Vergleich zu den anderen Pterinen bzw. im Vergleich zur Haut bedingt sein. Da die Anpassung an den Untergrund durch das Auge vermittelt wird (vgl. ODIORNE[1]), könnte eine nähere Analyse vielleicht Einblicke in die noch völlig unbekannten kausalen Zusammenhänge zwischen Bodenfarbe und Färbung des Tieres bringen.

IRMGARD ZIEGLER-GÜNDER*

Zoologisches Institut der Universität München, 5. Mai 1959.

Summary

Concomitantly with the morphological colour-change, larvae of Salamandra salamandra show a quantitative change in their pteridines. After being reared on a yellow background, all pteridines of the skin (isoxanthopterin and its derivatives, yellow pteridine, photosensitive pteridine) increase by about 1/3 as compared with the animals on a black background. In the retinal pigment-layer especially, the photosensitive pteridine is augmented.

* Jetzige Anschrift: Botanisches Institut der Technischen Hochschule Darmstadt.

[6] I. ZIEGLER-GÜNDER, Z. Naturf. *11b*, 493 (1956).

[7] Durch die Behandlung mit warmer $n/10$ HCl sind die Pterinkomponenten qualitativ gegenüber sehr milden Extraktionsbedingungen (z. B. $n/50$ NH_4OH) teilweise verändert. Eine quantitative Extraktion wäre aber in diesem Falle nicht möglich. Das gelbe und das photolabile Pterin sind jedoch unverändert erhalten.

[8] A. KÜHN: Naturwiss. *42*, 529 (1955).

A Suggested Mechanism for the Action of Choline Esters on Animal Organs, inferred from a Study of the Effect of Choline-, β-methylcholine-, and Thiocholine-esters

In previous works[1,2], we suggested that the rate-determining step in the action of a choline (Ch) ester on

[1] M. WURZEL, XXth Int. Congr. Physiol. Brussels 1956, Abstracts of Communications, p. 980.

[2] M. WURZEL and N. SAPEIKA, S. A. J. lab. clin. Med. *4*, 229 (1958).

Table I

Muscarinic effects and enzymatic hydrolysis / Choline-moiety kept constant, acyl changing

I. Hydrolysis by Enzyme		Ac	Prop	But	Benz
		True ChE			
Choline		1	1–3·7	100–200	∞
β-methylcholine		1	1·9	200<	∞
Thiocholine		1	1·2	125	∞
II. Potency on Organs		Muscarinic responses			
Choline	guinea-pig intestine	1	19	510	2,600
	cat blood pressure fall	1	37	2,900	110,000
β-methylcholine	guinea-pig intestine	1	24	4,500	500,000 <
	cat blood pressure fall	1	39	820	82,000
Thiocholine	cat blood pressure fall (atropine sensitive)	slightly active	inactive	inactive	inactive?

The figures for the enzyme are relative rates of hydrolysis obtained by dividing the rate of hydrolysis of the acetyl ester by the rates of hydrolysis of each of the other esters, thus the rate of hydrolysis of the acetyl ester is taken as unity.

The figures for the organs are relative equipotent molar concentrations obtained by dividing the equipotent molar concentration of each ester by that of the acetyl ester. Thus higher figures represent lower rates of hydrolysis and lower potencies respectively.

The figures for SCh ester hydrolysis were computed from KOELLE[6], except that for PropSCh.

a living organ might be its rate of hydrolysis by one of the cholinesterases (ChE). The experimental evidence for this was the observation that a series of choline esters of carboxylic acids, when applied to different animal organs, showed an order of potency parallel to the order of their rates of enzymatic hydrolysis, both with true ChE (in the case of 'muscarinic-') and with pseudo ChE (in the case of 'nicotinic-' effects). It was also shown[3,4] that when equipotent doses of different choline esters, i.e. concentrations causing half the maximum contraction of a muscle, are added to an identical concentration of true- or pseudo-ChE, they yield the same amount of reaction products (choline + carboxylic acid) per unit of time. For example: in the case of smooth muscle contraction, 500 molar concentration units of butyrylcholine (ButCh) are equipotent with 1 of acetylcholine (ACh); furthermore, 100–200 such units of ButCh give the same amount of end-products per minute as 1 unit of ACh, when hydrolyzed by an identical concentration of true ChE.

Carbaminoylcholine (CarbCh) is biologically as potent as ACh, but is not hydrolyzed by ChE-s. Since CarbCh is a carbamic acid ester, it differs from ACh only in having the CH_3-group of acetic acid replaced by a NH_2-group. It might therefore be thought that the receptor, i.e. the sensitive site on the organ, on which a compound added from outside acts, would be the same for both ACh and CarbCh. It might then be argued that as hydrolysis plays no part in the biological action of CarbCh, neither does it do so in the case of ACh.

According to WILSON and NACHMANSOHN[5], in the biological activity of carboxylic choline esters, 'hydrolytic activity is not a prerequisite for receptor activity'; however they also add that 'although there is no proof that such is the case, neither is there proof to the contrary'.

In our present investigation, the correlation between hydrolysis by ChE and biological potency was further studied with two additional series: β-methylcholine-(MeCh) and thiocholine-(SCh)esters. This choice was made since it is well known[7] that acetyl-β-methylcholine (AcMeCh) has predominantly muscarinic properties and weak nicotinic ones, and acetyl-thiocholine (ASCh) has strong nicotinic but barely discernible muscarinic effects, whereas ACh has both muscarinic and nicotinic properties[7].

Results. The enzymatic and biological effects of the above three sets of compounds are compared in Tables I and II. The choline- or choline-derivative moiety was kept constant, while the acyl part was in turn acetyl- (Ac), propionyl- (Prop), butyryl- (But), and benzoyl- (Benz). Despite the fact that the comparison was made between a homogenous enzyme solution on the one hand and highly organized biological structures on the other, the figures are on the whole in fairly close agreement. It may be concluded, therefore, that the orders of decreasing rates of enzymatic hydrolysis and of decreasing biological potency are parallel. Thus Table I and II strongly support the view that the rate-determining, perhaps the first, step in the biological action of these choline esters is their hydrolysis by one of the ChE-s.

In Tables III and IV, enzymatic hydrolysis rates and biological activity are compared, the acyl moiety being in all cases acetyl, and the alcohol moiety being in turn Ch, MeCh, and SCh. In this series no striking correlation can be detected between biological potency and rates of enzymatic hydrolysis: AcMeCh has a stronger muscarinic effect than could be foreseen from hydrolysis; ASCh is very weakly muscarinic, inspite of being a very good substrate for the ChE-s; the nicotinic potency of ASCh is high but perhaps less than would be expected from the rate of hydrolysis.

[3] M. WURZEL, Bull. Res. Council Israel *5A*, 303 (1956).

[4] M. WURZEL, submitted for publication A (1959).

[5] I. B. WILSON and D. NACHMANSOHN, in *Ion Transport Across Membranes* (by H. T. Clarke, Ed., Academic Press, New York 1954), p. 62.

[6] G. B. KOELLE, J. Pharmacol. exper. Therap. *100*, 160 (1950).

[7] D. BOVET and F. BOVET-NITTI, *Médicaments du système nerveux végétatif* (Ed. S. Karger, Bâle 1948), p. 375 and chapters VIII, IX, X.

Table II

Nicotinic effects and enzymatic hydrolysis / Choline-moiety kept constant, acyl changes

I. Hydrolysis by Enzyme		Ac	Prop	But	Benz
		Pseudo ChE			
Choline		1	0·5	0·4	3·4
β-methylcholine		1			
Thiocholine		1	0·6	0·7	11
II. Potency on Organs		Nicotinic responses			
Choline	frog rectus muscle	1	0·7	0·3	21
	cat-atropinized blood pressure rise	1	0·8	0·5	2·3
β-methylcholine	frog rectus muscle	1	2·1	2·1	57
	cat-atropinized blood pressure rise	inactive (complex responses)	inactive	active	active
Thiocholine	frog rectus muscle	1	3	2·3	<100
	cat blood pressure rise	1	1·1	0·6	2
	guinea-pig intestine, nicotinic contraction[9]	1	2	2·5	6

The figures: as described for Table I.

[9] M. WURZEL, submitted for publication B (1959).

The above data suggest the existence of two stages in the action of choline esters on animal organs: one dependent on ChE hydrolytic activity, and the second independent of ChE.

Stage A would consist of hydrolysis by one of the two ChE-s and would produce Ch, MeCh, or SCh as reaction products necessary for the next stage.

Stage B. The choline-derivative obtained by hydrolysis in stage A would react with the hypothetic receptor-component B, it too having its own substrate specificity: the muscarinic receptor is more sensitive to MeCh than to Ch, but is insensitive to SCh; the nicotinic receptor is sensitive to Ch and SCh, as well as to MeCh, but this latter is only available when high concentrations of AcMeCh are added, its hydrolysis by pseudo ChE of the 'nicotinic' organ being slower. While there is no doubt that the receptor-component of stage B has an easily definable substrate specificity, its nature is still a subject for speculation.

The apparent discrepancy, between the theory that ester hydrolysis is essential for biological activity and the fact that CarbCh is a stable but nevertheless potent compound, could be accounted for by assuming that CarbCh would by-pass stage A, directly entering the reaction at stage B. Its β-MeCh derivative was shown to have muscarinic, but no nicotinic effect, in analogy to the carboxylic ester of MeCh[6]. Thus, sensitivity to the β-methyl grouping seems to be characteristic of the receptor component of stage B, which is acted upon both by unhydrolyzed carbaminoyl-β-methylcholine and the hydrolyzable carboxylic acid esters of β-methylcholine.

As evidence of hydrolytic activity not being a prerequisite for receptor activity, WILSON and NACHMANSOHN[5] compared the behaviour of ButCh, ACh, and dimethylaminoethylacetate (tertACh) towards the enzyme-true ChE, with their action on the receptor. The above authors state that: (1) ButCh and ACh are both good stimulators of the receptor of the frog rectus muscle, but ButCh is a very poor true ChE enzyme substrate. They further claim (2) tertACh is a good substrate for the enzyme, but acts poorly on the receptor. On the other hand, our arguments, as deduced from Table I–IV, are:

Table III

Muscarinic effects and enzymatic hydrolysis
Choline-moiety changes, acyl constantly acetyl

I. Hydrolysis by Enzyme	ACh	AβMeCh	ASCh
	True ChE		
	1	5–7	0·7
II. Potency on Organs	Muscarinic responses		
Guinea-pig intestine	1	1·3	20≪
Cat blood pressure fall	1	1·4	100,000 <

The figures: as in Table I, except ACh being always taken as unity.

Table IV

Nicotinic effects and enzymatic hydrolysis
Choline-moiety changes, acyl constantly acetyl

I. Hydrolysis by Enzyme	ACh	AβMeCh	ASCh
	Pseudo ChE		
	1	72	0·23
II. Potency on Organs	Nicotinic responses		
Frog rectus muscle	1	180	20–40
Cat-atropinized blood pressure rise	1	certainly high	0·2
Guinea-pig intestine, nicotinic contraction	?	?	very potent, but ratio to ACh is not definable

The figures: as in Table I, except ACh being always taken as unity.

(1) the enzyme acting on the receptor of the frog rectus muscle is pseudo ChE (see Table II and [8]), therefore ButCh is a good substrate of the enzyme pseudo ChE and also a good receptor-stimulator;

(2) tertACh has little or no biological effect on the receptor, because its hydrolysis in stage A produces dimethylaminoethanol (tertCh) which probably has a low affinity for the stage B receptor-component (compare with Table III, where the acyl moiety is kept constant, while the choline moiety changes). Thus tertACh's behaviour is analogous to that of ASCh towards the muscarinic receptor, ASCh being a very good substrate for ChE-s, even though it has no affinity for the muscarinic receptor.

M. WURZEL

The Hebrew University, Hadassah Medical School, Department of Physiology, Jerusalem (Israel), June 5, 1959.

Résumé

Le mécanisme d'action des esters carboxyliques de la choline commence, probablement, par une hydrolyse enzymatique provoquée par une des cholinestérases. Les produits de l'hydrolyse réagissent avec un facteur inconnu, dont la spécificité est cependant définissable par la structure du résidu cholinique.

[8] J. DECHAVASSINE, Exper. *12*, 434 (1956).

Observations on Spontaneous Hemolysis in Shed Blood

The existence of a relation between the content of reduced glutathione of erythrocytes and their rate of spontaneous hemolysis has been suggested by various workers (LEMBERG and LEGGE[1]).

FEGLER[2] concluded from a study of horse blood that the rate of hemolysis bears a certain relation to the extent of oxidation of the reduced glutathione. KEILIN and HARTREE[3] found that the treatment of horse erythrocytes with sodium nitrite, though it produced complete oxidation of the reduced glutathione and converted the hemoglobin to methemoglobin, did not result in hemolysis of the erythrocytes until after 12 hours of storage in the cold. Since these two views appear to be mutually exclusive, a study of the relationship between the rate of hemolysis of nitrite treated and untreated erythrocytes and their glutathione content was attempted.

Blood samples from 10 Bali oxen (*Bos banteng*), 3 monkeys (*Macaca cynomolgus*), and 1 horse were collected in flasks containing potassium oxalate crystals, while those from 4 normal human subjects were collected in flasks containing heparin. The erythrocytes were washed with a 1% NaCl aqueous solution, 3–4 times with centrifuging. Aliquots were treated with equal volumes of an isotonic solution of $NaNO_2$ (1·06% in water) for 1–2 min and the nitrite was removed using the saline, washing 3–4 times, and centrifuging. The treated and untreated erythrocytes were washed twice with isotonic saline which contained: Streptomycin, 1 mg/ml, and 1000 units of penicillin, and then measured volumes of these cells were suspended, separately, in equal amounts of the isotonic saline. These cells were contained in centrifuge tubes, plugged with cotton wool and kept in a water bath at 37°C. The total hemoglobin and hemoglobin in the supernatant fluid were measured by a modification of the methode for plasma hemoglobin (DACIE[4]). Reduced glutathione was measured by the nitroprusside colour reaction of THOMPSON and WATSON[5]. The type of hemoglobin contained in the erythrocytes was determined by a paper electrophoretic technique (VELLA[6]). Determinations of reduced glutathione in the erythrocyte suspension and hemoglobin in the supernatant fluid were made at intervals of 0, 3, 6, 10, and 24 h.

The treatment of the erythrocytes with $NaNO_2$ oxidised the glutathione completely. In the untreated erythrocytes the content of reduced glutathione fell gradually to 8–40% (average: 20%) in ox, to 15–50% (average: 36%) in monkey, to 53% in the horse, and to 10–16% (average: 26%) in the human samples. The amount of hemoglobin in the supernatant fluid after 24 h at 37°C amounted to between 0·5 and 2·5% of the total hemoglobin at zero time. No significant differences were detected in the rates of spontaneous hemolysis in the nitrite treated and untreated erythrocytes from the same blood sample, though the rate of hemolysis appeared to be specific for each blood sample. The type of hemoglobin present (A, B, C, AB, or AC) in the samples from oxen did not appear to be related to the rate of spontaneous oxidation of the glutathione in the untreated cells or the rate of hemolysis of either the treated or the untreated samples. The hemoglobin in the horse, the monkey, and the human samples was electrophoretically homogeneous and normal in type for each species.

Spontaneous oxidation of reduced glutathione of shed blood is only one of many chemical changes taking place in red blood cells kept under conditions similar to those employed in these experiments. Loss of bicarbonate, conversion of glucose to lactic acid, loss of inorganic phosphate from breakdown of ester phosphate in the cells, loss of K^+ and gain in Na^+ (VARLEY[7]), loss of 'labile' iron and bilirubin (BARKAN and WALKER[8]) and of pyruvate (LONG[9]) are well known occurrences in the human shed blood.

The treatment of erythrocytes with $NaNO_2$ produces complete oxidation of reduced glutathione and converts oxyhemoglobin to methemoglobin but does not affect the catalase and the carbonic anhydrase activity (KEILIN and HARTREE[3]); it also denatures the globin of hemoglobin producing 'nitrite cat-hemoglobin' (BARNARD[10]). Methemoglobin formation *per se* does not appear to damage the erythrocytes (LEMBERG and LEGGE[1]).

From the present experiments, it appears that the rate of spontaneous hemolysis of erythrocytes from several species is independent of their content of reduced glutathione and is not affected adversely by the marked changes produced by treatment with nitrite.

Acknowledgment. I am grateful to Dr. LEE LIANG HIN of the Department of Bacteriology, Faculty of Medicine, University of

[1] R. LEMBERG and J. W. LEGGE, *Hematin Compounds and Bile Pigments* (Interscience Publishers, Inc., New York 1949), p. 517 and 523.

[2] G. FEGLER, Nature *170*, 624 (1952).

[3] D. KEILIN and E. G. HARTREE, Nature *157*, 210 (1946).

[4] J. V. DACIE, *Practical Haematology* (J. & A. Churchill Ltd., London 1956), p. 139.

[5] R. H. S. THOMPSON and D. WATSON, J. clin. Pathol. *5*, 25 (1952).

[6] F. VELLA, Nature *181*, 564 (1958).

[7] H. VARLEY, *Practical Clinical Biochemistry* (William Heinmann Medical Books, Ltd., London 1958), p. 7.

[8] G. BARKAN and B. S. WALKER, J. biol. Chem. *131*, 447 (1939).

[9] C. LONG, Biochem. J. *38*, 447 (1944).

[10] R. D. BARNARD, J. biol. Chem. *120*, 177 (1937).