bung°) ohne jegliche Verfärbung dar. Bei der Bildung dieser Säure handelt es sich um eine Ringerweiterung, wofür in der Literatur[1] schon einige Beispiele vorliegen.

Für die Ringerweiterung sprachen die Zinkstaub- und Natronkalk-Destillation der Säure IV, die immer erhebliche Mengen Indolderivate lieferten, die durch starke Fichtenspan- und positive Ehrlichsche Reaktion erkannt wurden. Daneben entstand auch Chinaldin; weiter können die folgenden Befunde nur aus der Konstitution IV gedeutet werden.

Wird IV mit Diazomethan behandelt, so erhält man die Verbindung $C_{17}H_{19}O_6N_3$, der die Konstitution V eines spiranartig verknüpften Pyrazolins mit einem Indolin zukommen muss. Wird dieser Tricarbonsäureester V im Hochvakuum destilliert, so erhält man nach heftiger Gasentwicklung ein Öl, das wir zur Kristallisation gebracht haben. Diese Verbindung vom Smp. 87° besass die Zusammensetzung $C_{16}H_{17}O_6N$ und stellt den der Tricarbonsäure IV entsprechenden Trimethylester VII dar. Der Beweis hierfür ist die Tatsache, dass die Verbindung VII beim Versetzen mit CH_2N_2 in quantitativer Ausbeute wieder die Substanz V liefert.

Bei der Destillation von V entsteht neben dem Ester VII noch ein Öl, der Zusammensetzung $C_{17}H_{19}O_6N$. Für diesen Körper schlagen wir die Formeln VIII bzw. IX mit Vorbehalt vor, weil nur aus ihnen die Tatsache interpretierbar ist, dass das Öl nach Verseifung mit HCl und anschliessender Natronkalkdestillation ausschliesslich 2,3-Dimethylchinolin XII liefert. Eine Verbindung, nach III formuliert, wird einem solchen Reaktionsverhalten nicht gerecht. Die Bildung der Produkte der thermischen Zersetzung von V im Hochvakuum haben eine weitgehende Ähnlichkeit mit den in der Literatur[2] beschriebenen Reaktionen von α-Methylzimtsäure und Diazomethan.

Für das Vorliegen einer o-Aminozimtsäurestruktur gemäss IV bzw. VII sprachen auch UV.-spektroskopische Beobachtungen, die an anderer Stelle näher erläutert werden.

Die saure Hydrolyse von VII ergab nach Behandlung des Hydrolysats mit CH_2N_2 Chinaldin-4-carbonsäuremethylester X. Überraschenderweise entsteht dieser Körper nicht über die Chinaldin-4,8-dicarbonsäure VI, die bei der Hydrolyse unter den gleichen Bedingungen völlig unverändert bleibt. Es kann also die dem Ester X entsprechende Säure nicht aus der Chinolinstruktur VI hervorgegangen sein. Einige weitere Nebenbefunde wollen wir in der demnächst erscheinenden ausführlichen Arbeit näher behandeln.

Ein wichtiger Befund für das Vorliegen eines Indolderivates in IV und VII konnte weiter durch die Ozonisierung des kristallisierten Trimethylesters VII erbracht werden; man erhielt hierbei Isatin-7-carbonsäuremethylester XI. Wenn über die durchlaufenen Stufen bis zur Isatinbildung nichts Genaues gesagt werden kann, so halten wir die Bildung von XI doch für strukturbeweisend. Eine weitere Stütze für unsere Formulierung stellt die Photodimerisation des Esters VII dar. Bei der Bestrahlung des Esters VII mit UV.-Licht fällt ein kristallisierter Körper der Zusammensetzung $C_{32}H_{34}O_{12}N_2$ (Smp. 232°) an, der im Hochvakuum nicht mehr flüchtig ist. In Analogie zu den Bestrahlungsprodukten der trans-Zimtsäure ist das Dimerisat voraussichtlich ein Cyclobutanderivat XIII mit α-Truxillsäurestruktur.

Alle aus der Tricarbonsäure IV durch die verschiedensten Reaktionen erhaltenen Chinolinderivate möchten wir nach diesen Befunden als Artefakte ansehen.

Herrn Dr. OTTO SVIERAK möchte ich für seine Mitarbeit bestens danken.

K. EITER

II. Chemisches Laboratorium der Universität Wien, den 6. Januar 1955[1].

Summary.

Using series of different reactions it has been possible to demonstrate that the tricarboxylic acid arising from anthranilic acid and pyruvic acid has not the constitution I but is correctly represented by IV. Several derivatives of this tricarboxylic acid were shown to have formulae that are in sharp contrast to those found in literature.

[1] E. WENKERT und TH. L. REID, Exper. *10*, 417 (1954). – TH. WIELAND, O. WEIBURG, E. FISCHER und G. HÖRLEIN, Ann. Chem. *587*, 149 (1954). – P. L. JULIAN, H. C. PRINTY, R. KETCHAM, R. DOONE, J. Amer. Soc. *75*, 5305 (1953). – L. HORNER, Ann. Chem. *548*, 117 (1941). – G. JACINI, Gaz. Chim. *73*, 85 (1943). – G. R. CLEMO und H. VIPOND, Chem. and Indust. *1949*, 856.

[2] K. v. AUWERS und E. CAUER, Ann. Chem. *470*, 2956 (1929).

[1] Eine ausführliche, aber mit vorliegender Mitteilung inhaltlich identische Arbeit ging der Redaktion bereits am 21. Oktober 1954 zu.

Morphogenetic Effects of Centrifugation on the Isolated Ectoderm and Whole Embryo of Some Anurans[1]

In certain amphibians, a secondary tail is formed when the embryos are subjected to centrifugation[2]. Recently PASTEELS[3] reported that in some species the secondary tail was produced at high frequency only when late blastulae or early gastrulae were centrifuged under proper conditions. The secondary tail thus formed contained spino-caudal structures, such as notochord, myotome and spinal cord, and was often completely separated from the primary axis. Furthermore PASTEELS isolated the ectoderm, which was folded during the centrifugation of whole embryos, and cultured it *in vitro* or transplanted it to another embryo. Results were presented which indicated the differentiation of spino-caudal and other axial structures from this ectodermal piece. From these experiments, he suggested that the centrifugation can directly affect the presumptive ectoderm and initiate the axial differentiation in it[3]. In order to establish such a possibility, it seems necessary to make an experiment, in which the presumptive ectoderm is previously isolated and then centrifuged. Such experiments, carried out on the embryos of *Bufo vulgaris*, *Rana japonica* and *R. nigromaculata* are reported in this paper with some other related experiments.

[1] Supported by a grant from the Rockefeller Foundation, the Scientific Research Found of the Ministry of Education, and the Asahi Grant for Scientific Research.

[2] A. M. BENTA and R. A. GORTNER, J. exp. Zool. *18*, 433 (1915). – M. BAGINI, Arch. ital. Anat. Embryol. *22*, 35 (1925). – P. PASQUINI and G. REVERBERI, Boll. Ist. Zool. R. Univ., Roma. *7*, 1 (1929). – H. W. BEAMS, R. L. KING, and P. L. RISLEY, Proc. Soc. exp. Biol. N.Y. *32*, 181 (1934). – I. MOTOMURA, Sci. Rep. Tohoku Imp. Univ. Biol. *10*, 211 (1935).– A. M. SCHECHTMAN, Proc. Soc. exp. Biol., N.Y. *37*, 153 (1937). – J. PASTEELS, Arch. Biol. Paris *51*, 355 (1940). H. BAN, Zool. Mag. (in Japanese) *62*, 112 (1953).

[3] J. PASTEELS, Exper. *3*, 30 and 73 (1947); Acta Anatomica *4*, 219 (1947); J. Cyto-embryol. belgo-neerland. Gand 88 (1949); J. Embryol. exp. Morph. *1*, 5 and 125 (1953).

I.–*The Conditions of Centrifugation Necessary for Obtaining the Secondary Tail in the Whole Embryo.* Some preliminary experiments were run in order to determine the stage and centrifugal force optimal for obtaining secondary structures by centrifugation. The temperature at the time of centrifugation was chosen according to that of the breeding season of each species used: 5°C for *Rana japonica*, 10°C for *Bufo vulgaris*, 20°C for *Rana nigromaculata*. The optimal centrifugal force was found to be between 350 and 400 g for *Bufo vulgaris*, and between 500 and 800 g for *Rana japonica* and *R. nigromaculata* for duration of 5 min.

Of all factors, the stage of centrifugation seems to be most important. The type of abnormality and site of secondary axis varied according to the stage of centrifugation. On centrifuging at the earliest stage of gastrulation, i.e. at the time of appearance of pigment in the future blastoporal area, well developed secondary tails were often formed from the medio-ventral region of the

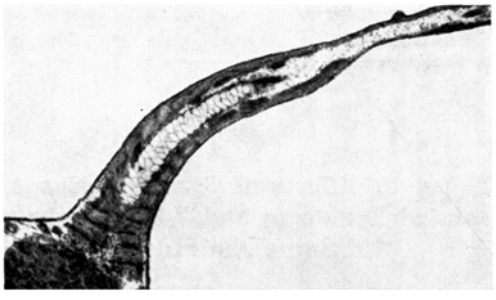

Fig. 1.–A secondary tail produced by centrifugation of whole embryo of *Rana japonica*, located medio-ventrally in the trunk. Notochord, myotome, spinal cord, pronephros and mesenchyme are visible (about 40 ×).

embryo. Centrifugation at a little earlier or later stage gave rise to secondary structures located in the head region. On centrifuging still earlier, or still later, only head malformations were obtained but no secondary tails. When embryos were centrifuged still later, i.e. shortly after the beginning of actual invagination, neither secondary tail nor head malformation could be produced, and the subsequent development of the embryo was completely normal. Thus it is obvious that

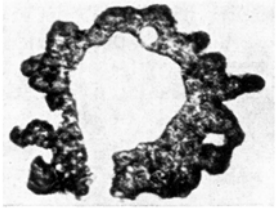

Fig. 2.–Centrifuged ectodermal explant of *Rana japonica*. Differentiation of epidermal cells (about 50 ×).

the reactivity of the embryo to form supernumerary structures under centrifugation is strictly limited to a short period of from one to two hours duration. In the embryo of *Rana japonica* and *Bufo vulgaris*, a well-defined secondary axis could be obtained in some instances at the frequency of 100%. According to histological examinations, the majority of the secondary axis contained spino-caudal structures, such as notochord, myotome, and spinal cord. Some of these were perfectly separated from the primary axis. In *Rana nigromaculata*, the frequency of double-tailed embryos was below 30% and the differentiation of the accessory tissues was not well advanced as in other species. In general, the results of this series of experiments confirmed the above cited data of PASTEELS.

Fig. 3.–A secondary tail produced by centrifugation of whole embryo of *Bufo vulgaris*. A large notochord with myotome, spinal cord, and pronephros (about 40 ×).

II.–*Centrifugation of the Isolated Ectoderm.* The presumptive ectoderm was isolated from the latest blastula immediately before the appearance of the pigmented spot at the position of the future blastopore. Two isolated pieces were fused with their inner surfaces facing each other. Operation, centrifugation and culture were done in HOLTFRETER solution buffered at pH 7·0. The explants thus obtained were placed in a sealed tube and centrifuged under sterile conditions. As control, intact embryos from the same batch and of the same stage were centrifuged simultaneously with the explants.

Fig. 4.–Centrifuged ectodermal explant of *Bufo vulgaris*. Epidermal cells and sucker cells (about 40 ×).

The conditions established in series I were followed as strictly as possible. It was observed that the explants were remarkably flattened by centrifugal force, but were never folded as in the case of the whole embryo (see above). The explants were cultured for from 7 to 9 days at 20°C and studied in sections. As shown in Table I, the centrifuged, isolated ectoderm of *Bufo vulgaris* and *Rana japonica* differentiated the epidermis and occasionally the sucker cells, but it did not show any mesodermal or neural differentiation and could not be distinguished from the untreated ectodermal explants either by histological or external observations. That the isolate of the presumptive ectoderm of anuran gastrula differentiates sucker cells was well established by earlier experiments[1]. The centrifuged whole embryos of the

[1] T. YAMADA, J. Facul. Sci., Tokyo Imp. Univ. *3*, 239 (1933). – J. HOLTFRETER, Roux' Arch. *129*, 669 (1933). – T. YAMADA, J. Facul. Sci., Tokyo Imp. Univ. *5*, 133 (1938). – L. RAUNICH, Monitore Zool. Ital. *8*, 11 (1942).

Table I.–Effects of centrifugation of isolated ectoderm and whole embryo

Species	Batch	Stage*	Centrifugal force** g	Centrifuged explant		Centrifuged whole embryo				
				Usable cases	Neural or mesodermal tissues	Usable cases	Secondary tail	Head malformation	Subnormal	Normal
Bufo vulgaris	A-1	I	400	14	0	22	19	1	2	0
	B-1	I	350	10	0	21	17	2	2	0
	B-2	I	500	7	0	18	12	4	2	0
	C-1	0	350	9	0	22	12	3	5	2
	C-2	0	1800	9	0	—	—	—	—	—
				49	0	83	60	10	11	2
Rana japonica . . .	A-1	II	500	9	0	30	9	12	7	2
	A-2	II	800	7	0	25	7	12	4	2
	B-1	I	800	8	0	27	1	0	4	22
	C-1	I	800	4	0	41	11	11	10	9
	C-2	I	500	8	0	38	2	3	23	10
				36	0	162	30	38	48	45

* Stage 0: The latest blastula immediately before the onset of gastrulation. Stage I: The earliest gastrula at the moment of appearance of the pigmented spot in the blastoporal region. Stage II: The succeeding stage with the small pigmented patch.

** The length of time of centrifugation is 5 min respectively.

control series differentiated the secondary axis at a frequency higher in *Bufo vulgaris* (60 out of 83 cases) than in *Rana japonica* (30 out of 161 cases). In *Rana nigromaculata* (data not shown in the table), the isolated ectoderm was centrifuged at 250 to 2000 g. Differentiation of epidermis and sucker cells but no other tissue was observed in 105 thus treated explants. However, as the frequency of the secondary axis formation in the simultaneously centrifuged whole embryo of the control series was very low, this negative result was not as convincing as those of experiments on *Bufo vulgaris* and *Rana japonica*.

The results of series II considered together, do not support the idea of direct activation of the presumptive ectoderm by the centrifugal force, previously mentioned.

III.–*Subcytolytic Treatment of the Presumptive Ectoderm.* A close similarity of the basic mechanism of the subcytolytic neutralization of the ectoderm isolate and that of the secondary axis formation by centrifugation was suggested by PASTEELS[1]. However, the published experiments[1] of subcytolytic neuralization were limited to *Triturus*, which is known to be non-reactive toward centrifugation. Furthermore, this subcytolytic treatment was found to lead to the formation of unorganized neural tissue and archencephalic structures such as brain, eye, nose and mesenchyme, but not to the formation of spino-caudal structures such as notocord, somites, and spinal cord, which are encountered in our secondary axis[2]. For this reason, it is worth-while to investigate the morphogenetic effect of subcytolytic stimulus on the ectoderm of the amphibians, whose embryos are known to react to centrifugation.

The presumptive ectoderm of early gastrula of *Rana nigromaculata* was isolated and exposed to a solution of ammonia (pH 12·0). Out of 41 aggregates thus obtained, 16 differentiated the mesenchyme and neural tissue. The other structures differentiated were epidermis and sucker cells, both of which occurred also in the control

[1] J. PASTEELS, Exper. *3*, 30 and 73 (1947); J. Cyto-embryol. belgo-neerland. Gand 88 (1949); J. Embryol. exp. Morph. *1*, 5 and 125 (1953).

[1] J. HOLTFRETER, J. exp. Zool. *98*, 161 (1945). – T. YAMADA, Biol. Bull. *98*, 98 (1950 a).

[2] By subcytolytic treatment, myotomes and notochord were obtained in the isolate from the ventral marginal zone of *Triturus*-gastrula, which without the treatment differentiated the blood island, mesothelium and pronephric tubules (YAMADA, 1950a).

Table II.–Histological observation of abnormality of *Bufo vulgaris* caused by centrifugation (cultured for 6 days at 20°C)

Batch	Centrifuged embryo	Usable cases	Abnormality of primary axis			Histological constitution of secondary axis										
			Malformation of brain	Chordarization of brain	Malformation of notoch.	Otic vesicle	Brain	Neural tube	Notochord	Myotome	Pronephros	Mesenchyme	Blood cells	Fin	Sucker	Gut
A-1	24	22	3	0	0	2	7	13	3	13	1	19	4	4	1	2
B-1	26	21	8	2	3	1	5	8	7	8	1	15	5	1	1	2
B-2	30	18	10	3	3	2	7	9	7	9	0	12	10	3	2	3
C-1	25	22	7	3	0	4	5	8	8	9	1	11	4	2	1	4
	105	83	28	8	6	9	24	38	25	39	3	57	23	10	5	11

series. In another series[1], the presumptive ectoderm of early gastrula of *Rana pipiens* was isolated and treated with 0·2 M borate buffer at pH 9·3 for 6–12 min. One hundred and sixty-six isolates thus treated were allowed to reaggregate into 20 larger explants, and cultured at 18°C for 4 to 8 days. Eighteen pieces of such aggregates could be studied in sections, and 23 neural structures were recognized, of which 7 were classified into the brain-type and 2 into the nose-type[2], while others were unorganized neural fragments. All aggregates contained extensive groups of sucker cells as well as epidermis and neural cells. Without close comparative study, the large vacuoles of sucker cells could have been mistaken for notochord vacuoles.

The results of this series do not support the assumption that the secondary axial system in question is caused by a mechanism similar to subcytolytic stimulation of the isolated ectoderm, by showing that the latter causes only archencephalic type or unorganized neural differentiation, and not the spino-caudal type encountered in the secondary axis produced by centrifugation.

The data presented here seem to throw doubt on the assumption that a simple, direct activation of individual cells of the presumptive epidermis by centrifugation is the cause of the formation of a secondary axis. However, the possibilities are not excluded that the centrifugal force affects the presumptive ectoderm directly when it is within the whole intact embryo, perhaps by changing its relationship with other components of the embryo.

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Zusammenfassung

Zentrifugierung der Froschkeime gerade vor oder am ersten Anfang der Gastrulation bringt bekanntlich ein sekundäres axiales System an der ventralen oder lateralen Flanke der Embryonen hervor. Um die Annahme zu prüfen, dass diese spino-kaudalen Gebilde aus dem präsumptiven Ektoderm durch eine direkte Wirkung der Zentrifugierung hervorgehen, wurde isoliertes, präsumptives Ektoderm unter den genau kontrollierten Bedingungen zentrifugiert und gezüchtet. Die Isolate differenzierten Epidermis und Haftdrüsenzellen, aber kein axiales Gewebe. Subzytolytische Behandlung des isolierten, präsumptiven Ektoderms der jungen Gastrulae brachte unorganisierte Neuralgewebe oder archenkephale Gebilde, aber kein spino-kaudales Gewebe hervor. Also gaben die beiden Versuchsreihen keine Unterstützung für die oben genannte Annahme.

[1] The experiment was performed at the Institute for Cancer Research and Lankenau Hospital Research Institute in Philadelphia, by one of the writers, YAMADA, when he was there as a guest investigator. He expresses his sincere thanks to Dr. R. BRIGGS and other members of the Institute for their kind help.

[2] T. YAMADA, Embryologia *1*, 1 (1950b).

On Phosphorylation in Brain Tissue in the Chick

Experiments in the literature relate P_{32} exchange in tissue to the biosynthesis of adenosine triphosphate, a recent report by KREBS[1] presents an approach which differs from those previously employed. In his experiments use is made of sufficiently large quantities of adenosinetriphosphate and inorganic phosphate as a carrier for the label that any fate of the isotope beyond that of its exchange with the adenosine phosphates is negligible in the dilute enzyme system employed. In addition, the organic phosphates are separated by paper chromatography in order that the fate of the label within this system can be well ascertained. However, SLATER and HOLTON[2] believe that the kinetic treatment of KREBS was over simplified. Their criticism was based on an argument involving the system:

$$(1)\quad \text{Alpha-Kg} + \text{CoA} + \text{DPN}^+ \xrightarrow{k_1} \text{Succinyl-CoA} + \text{DPNH} + \text{H}^+.$$

$$(2)\quad \text{Succinyl-CoA} + \text{ADP} + \text{H}_3\text{PO}_4 \underset{k_3}{\overset{k_2}{\rightleftarrows}} \text{Succinate} + \text{CoA} + \text{ATP}.$$

Essentially, if k_2 and k_3 are large with respect to k_1, then exchange of ATP with inorganic phosphate will not measure ATP synthesis. Although the technique might not be sufficiently critical in experiments designed to search for errors in P/O ratios, we believed that such an approach could be suitable to compare tissues with respect to P_{32} exchange. Reported below is an application of the above experimental design in its simplest aspects. P_{32} exchange was examined in liver and brain homogenates from these tissues of the chick embryo.

Three 17 day chick embryos and a day old hatched chick were used for each experiment. The brain and liver were rapidly removed, weighed and placed in beakers containing ice cold saline. These beakers were stored for fifteen minutes at freezing temperatures, homogenized, and sufficient saline was mixed with the homogenate so that for each gm of tissue there would be 20 ml of saline. 4 ml each of liver and brain suspensions were incubated with 12 mg of commercially prepared ATP and 0·2 ml of P_{32} as KH_2PO_4 in a Dubnoff metabolic shaker. At the end of the times indicated below (Table) 1 ml of the enzyme suspension was removed from the beaker, placed into a 15 ml conical centrifuge tube, and the enzyme activity was stopped by adding 0·2 ml of 30% trichloracetic acid. The centrifuge tubes were placed in an ice bath and centrifuged at 5° for 15 min. The supernatant solutions were frozen and stored for the chromatographic separation of the labeled components. When the chromatograms were complete, the paper was cut into one-fourth inch strips after being numbered consecutively, so that the strip having the number one was nearest the origin, and the strip having the highest number was nearest the solvent front. Each strip was then measured for radioactivity using a Geiger tube with an efficiency of approximately 2%. After this measurement each strip was placed in a test tube, 5 ml of water were added, and the optical density at 260 mμ was recorded using the Beckman Model DU spectrophotometer.

[1] H. A. KREBS, A. RUFFO, M. JOHNSON, L. V. EGGLESTON, and R. HEMS, Biochem. J. *54*, 107 (1953).

[2] E. C. SLATER and F. A. HOLTON, Biochem. J. *56*, 28 (1954).

[3] L. V. EGGLESTON and R. HEMS, Biochem. J. *52*, 156 (1952).