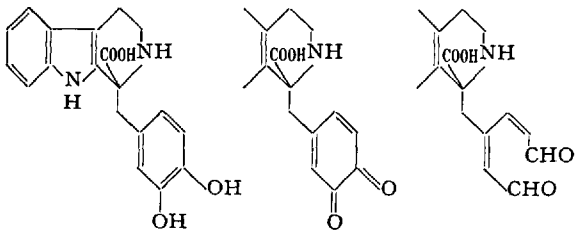
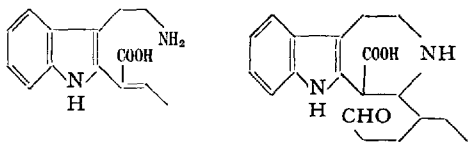
The Biogenesis of the *Tabernanthe Iboga* Alkaloids*

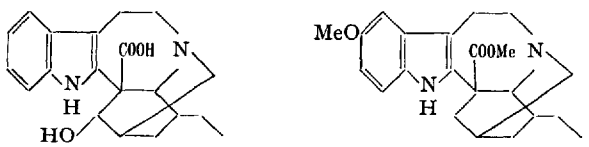
In recent years much progress has been made on the part of chemists to develop a set of working hypothesis by which the biosynthesis of indole alkaloids might be explained in broad outline[1]. In this way the structures of such apparently unrelated compounds as strychnine, yohimbine, ajmaline and cinchonine have been rationalized. Since the ring system of the major *Tabernanthe iboga* alkaloids, ibogaine, ibogamine and tabernanthine, has now been shown to be that in (VIII)[2], it seemed appropriate to consider how it might be possible to account for its formation based on the existing theories.



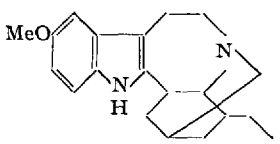
(I) (II) (III)



(IV) (V)



(VI) (VII)



(VIII)

A plausible route starts from the α-type condensation product (I) derived from tryptophan and 3:4-dihydroxyphenylalanine. Ring C can become seven membered as a consequence of an elimination followed by readdition of the basic nitrogen, activation for the first step being provided by a carbonyl group either in an *o*-quinone [such as (II)] or in the Woodward fission product (III). The Michael type ring closure of (IV) in the second step to finally yield (V) requires little comment. Mannich condensation with formaldehyde and ring closure completes the elaboration of the skeleton (VI), whose conversion into voacangine[2] (VII) or ibogaine (VIII) offers no difficulty[3]. One is lead to speculate on the possible occurrence of alkaloids similar to (VI) which would contain a hydroxyl in the position shown. The high molecular weight Voacanga alkaloids, e.g., voacamine[4] and voacorine[5] may well arise from intermediates in such a biogenetic scheme as suggested above.

W. I. TAYLOR

CIBA Pharmaceutical Products Inc., Summit, N. J., USA., July 23, 1957.

Zusammenfassung

Die vorliegende Arbeit schlägt eine Biogenese der Tabernanthe-Alkaloide vor, die von einem α-Kondensationsprodukt von Tryptophan mit 3,4-Dioxyphenylalanin ausgeht. Der siebengliedrige Ring C dieser Alkaloide entsteht durch Ringöffnung und erneute Ringschliessung eines Oxydationsproduktes. Weitere Stufen umfassen eine Mannich-Kondensation, eine Reduktion und einen Ringschluss zu einer Hydroxycarboxyverbindung, aus der durch einfache Reaktionen Voacangin und Ibogain abgeleitet werden können.

* The Alkaloids of *Tabernanthe Iboga*, Part V.

[1] For a recent summary see R. ROBINSON, *The Structural Relations of Natural Products* (Oxford at the Clarendon Press, 1955), p. 100.

[2] W. I. TAYLOR, J. Amer. chem. Soc. *79*, 3298 (1957).

[3] It is already known that voacangic acid suffers decarboxylation in acidic media to furnish ibogaine [M.-M. JANOT and R. GOUTAREL, C. R. Acad. Sci. Paris *241*, 986 (1955)]. Compare the analogous decarboxylation of 2-substituted 2-carboxy-β-carbolines [G. HAHN, L. BÄRWALD, O. SCHALES, and M. WERNER, Ann. *520*, 107 (1935)].

[4] R. GOUTAREL, F. PERCHERON, and M.-M. JANOT, C. R. Acad. Sci. Paris *243*, 1670 (1956).

[5] R. GOUTAREL and M.-M. JANOT, C. R. Acad. Sci. Paris *242*, 2981 (1956).

Hydroxyproline Production in Chick Fibroblast Cultures

Introduction.—While it is well known that argyrophil fibres develop in fibroblast cultures, histological methods provide at best an inadequate quantitative assessment of their production. Hydroxyproline is an imino acid found in collagen, reticulin and in certain soluble substances which are probably their precursors (HARKNESS *et al.*[1], OREKHOVITCH *et al.*[2]). We thought it of interest to measure quantitatively the production of hydroxyproline in chick aorta cultures as an index of their formation of extracellular fibre material. 18-day embryos were used with culture periods of 10, 21 and 28 days.

It has been suggested (STETTEN[3]) that proline is a precursor of hydroxyproline, and we have further assessed the effect of proline enrichment on hydroxyproline formation.

Materials and Methods

Cultures.—White Leghorn eggs were used and incubated at 38°C for 18 days. Embryos were removed under aseptic conditions, the thorax opened, and the heart and proximal parts of the great vessels removed. The latter were cut into small pieces of about 1 mm³ for culture. Cultures were continued to 10, 21 and 28 days.

[1] R. D. HARKNESS, A. M. MARKO, H. M. MUIR, and A. NEUBERGER, Biochem. J. *56*, 558 (1954).

[2] N. N. OREKHOVITCH, K. D. TUSTANOVSKY, K. D. OREKHOVITCH, and N. E. PLOTNIKOVA, Biochimia *13*, 55 (1948).

[3] M. R. STETTEN, J. biol. Chem. *181*, 31 (1949).

For 10-day cultures 2 eggs were used at a time to provide a pool of 40–80 inocula. Half of these were weighed and estimated at once for hydroxyproline. The remainder were cultured in hollow slides in groups of 10 with a drop of 50% fowl plasma and the same volume of 33% chick embryo extract containing 50 μg/ml of added ascorbic acid. At 10 days, culture-bearing areas were excised from the plasma clot, weighed, and estimated for hydroxyproline, as was the remaining plasma.

21-and 28-day cultures were made from pools of about 150 innocula obtained from four embryos. By the use of a table of random numbers 30 of these innocula were selected for immediate determination of hydroxyproline, and were weighed together in a sealed bottle. 60 of the remaining pieces were cultured in groups of 10 in Carrel flasks in a medium consisting of 50% fowl plasma and 50% by volume of 10% chick embryo extract. Ascorbic acid was added to the medium to produce a final concentration of 50 μg/ml. The remaining 60 pieces were treated in a similar way with the exception that the medium in each flask contained 5 μg of added proline.

After 5 or 6 days 0·5 ml of 10% embryo extract containing 25 μg ascorbic acid, was added to each flask. The extract for those in which the effect of proline was tested contained in addition a further 5 μg of added proline. At 10–12-days and subsequently at 5–6-day intervals, the extract was renewed. The supernatant fluid phase was removed from the flasks for hydroxyproline estimation before addition of new extract. At 21 or 28 days, supernatant fluid was again removed for hydroxyproline analysis, and culture containing areas were cut from the plasma clot. Their hydroxyproline content was determined, as was that of the plasma clot remaining after their excision. Thus each pool of about 150 inocula provided cultures analysed at 21 and 28 days, half of which were enriched with proline.

Initial determinations of the hydroxyproline content of embryo extract and plasma were made for all experiments. Four or six Carrel flasks were also incubated as controls for each 21–28-day experiment, half with and half without added proline, but containing no cultures. Embryo extract was added to these controls and supernatant removed for analysis as already described.

Chemical Method.—After colour development by the method of NEUMAN and LOGAN[4] hydroxyproline content was determined by absorption measurements in a Zeiss spectrophotometer over the 540–640 mμ range. Maximum absorption was at 560 mμ and readings were corrected for general density of the sample following LOTHIAN[5]. The validity of this procedure was checked by recovery experiments in which known amounts of standards were added to embryonic extract.

Proline was obtained from Hoffmann-La Roche. A solution containing 50 μg/ml was prepared and sterilised by filtration, and estimated for contamination by hydroxyproline.

Calculation of Results.—For 10-day cultures, increases in hydroxyproline were found in both the excised culture bearing areas and in the remaining plasma. In addition, for 21-and 28-day cultures, hydroxyproline was found in the supernatant.

Total hydroxyproline production ascribed to the cultures was obtained by summation of values from these sources, with the necessary deduction of any contribution due to the initial plasma.

The proportionate weights of cells and plasma excised were unknown. At worst plasma could have constituted the whole weight of excised culture area, and we have consequently, in the cases where hydroxyproline was detected in the initial plasma, deducted from the total the amount of hydroxyproline contained in a weight of such plasma equal to that of the excised areas.

Results

As was to be expected, no hydroxyproline was produced as a consequence of incubation of plasma and embryo extract. The proline used was found to contain about 2% hydroxyproline as an impurity, and results have been corrected accordingly. Mean recovery of known amounts of hydroxyproline added to embryo extract to assess the reliability of the chemical method was 97·5 ± 5·0% for 27 estimations.

10-Day Cultures.—10 experiments were done in all. The numbers of cultures made in each of these ranged from 40 to 150, and for simplicity of presentation all results given subsequently in the text are expressed in terms of 10 cultures.

On this basis, inocula investigated before culture as controls had a mean wet weight of 10·5 ± 0·7 mg, and a hydroxyproline content 2·54 ± 0·35 μg. Culture-bearing areas excised from the plasma clot after 10 days cultivation had a mean wet weight of 92·9 ± 4·2 mg, and a hydroxyproline content of 4·76 ± 0·5 μg.

Of the five batches of plasma used to make up the media, only one contained detectable amounts of hydroxyproline, but at 10 days the plasma remaining after excision of the culture areas contained appreciable amounts (3·13 ± 0·73 μg).

After appropriate deduction for initial plasma hydroxyproline as described in *Methods* mean hydroxyproline increase in the culture areas was 2·27 ± 0·56 μg and in the remaining plasma 2·57 ± 0·56 μg. Thus of the total hydroxyproline increase of 4·84 ± 1·26 μg, some 53% was found in the plasma remaining after the culture-bearing portions had been excised.

21- and 28-Day Cultures.—Six experiments were done, and in all 635 cultures were made. In the text results are again presented in terms of 10 cultures, and on this basis the mean wet weight of control inocula was 12·87 ± 0·67 mg, and their hydroxyproline content 5·07 ± 0·55 μg.

In those cultures continued to 21 days, culture-bearing areas excised from the plasma clot had mean wet weights of 147·72 ± 22·67 and 119·35 ± 26·89 mg respectively for material with and without proline enrichment. Corresponding hydroxyproline means were 10·63 ± 1·77 and 9·47 ± 0·90 μg, and in neither case do these means differ significantly.

In half the experiments the plasma used contained measurable amounts of hydroxyproline, but that remaining after excision of the culture-bearing areas showed no consistent pattern of hydroxyproline increase. Indeed, in the two cases the plasma hydroxyproline content diminuished slightly. This was not true, however, of the supernatant fluid, which in all cases contained appreciable hydroxyproline when removed from the cultures whereas it contained none when prepared as embryonic extract. The mean hydroxyproline content of supernatant removed from proline-enriched cultures was 7·5 ± 1·3 μg and from non-enriched cultures 6·2 ± 0·7 μg. The mean total increase for cultures with added proline, corrected for initial plasma hydroxyproline (see *Methods*) was 15·13 ± 1·96, and for the remainder 11·93 ± 2·35 μg.

The results from comparable 28-day cultures follow a similar pattern. Mean wet weight of excised material was 126·06 ± 23·2 mg for proline-enriched cultures, and their hydroxyproline content was 11·30 ± 0·85 μg. Cul-

[4] R. D. NEUMANN and M. A. LOGAN, J. biol. Chem. *186*, 549 (1950).
[5] G. F. LOTHIAN, *Absorption Spectrophotometry* (Hilger and Watts, London 1949).

ture areas without added proline had a mean weight at excision of 156·80 mg, and a hydroxyproline content of 8·33 ± 0·46 µg. As in the 21-day cultures, the plasma increase was slight but the supernatant removed during culture had 7·32 ± 0·68 µg of hydroxyproline in proline-enriched cultures and 7·78 ± 0·61 in the remainder. The overall increase in hydroxyproline attributable to the cultures was 14·7 ± 1·11 µg with added proline, and 13·1 ± 1·21 without addition.

Thus for both 21- and 28-day cultures, although corrected hydroxyproline recovery was slightly higher when proline was added to the medium, the increase was not significant, and neither does it become so if 21- and 28-day cultures are grouped together for comparison.

Of the total hydroxyproline increase, about 50% was found in the supernatant fluid phase in both 21-and 28-day cultures, a distribution closely resembling that found in 10-day material.

Discussion

The application of NEUMAN and LOGAN's[4] technique to plasma containing minimal amounts of hydroxyproline may be misleading if absorption measurements are made, as they suggest, only at 560 mµ. Although there appears to be no reference to this problem in the literature, we found on occasions non-specific absorption at 560 mµ, shown by the fact that there was no peak at this wavelength if measurements were made over the range from 540–640 mµ. Consequently in our experiments hydroxyproline estimations, as well as being duplicated, were derived from absorption curves over the 540–640 mµ range. (At these limits standard hydroxyproline solutions show zero absorption.) It was then possible to correct absorption peaks at 560 mµ for non-specific absorption in the customary manner.

It is probable that our figures for total hydroxyproline production by cultures are slightly underestimated, since we have subtracted from our totals the amount of hydroxyproline contained in a weight of initial plasma equal to that of the excised culture areas (see 'Calculation' of *Results*). Clearly the weight of the excised areas was in part attributable to the cells they contained, although it was impossible to determine the relative proportion of cells and plasma. Our correction, however, although excessive, excluded the possibility of overestimation.

Again, recovery experiments showed our method to give slightly reduced values for hydroxyproline (97·5%).

It is by now well established by ordinary histological means (e.g. MAXIMOV[6]), by electron microscopy (e.g. PORTER and VANAMEE[7]) and by chemical techniques (GERARDE and JONES[8]) that collagen is a product of the metabolic activity of fibroblasts, and consequently some increase in hydroxyproline in culture was to be expected. In our experiments, however, some 50% of the total hydroxyproline was found in the supernatant embryo extract removed from 21- and 28- day cultures, while most of the remainder was present in the excised culture-bearing areas. Thus, at least 50% of the hydroxyproline produced was in a freely diffusible form. It is possible that this finding indicated the presence of soluble forms, or even precursors, of collagen (HARKNESS[1], OREKHOVITCH[2], JAMES[9]). The further possibility, that collagen fibres should be deposited at some distance from the cells via a soluble intermediary, receives some support from the literature (DOLJANSKI and ROULET[10]), and it is known that collagen can be precipitated from solution (VANAMEE and PORTER[11]), although electron microscopic studies suggest fibril formation both within, and in the immediate vicinity of, the cell (JACKSON[12]).

In the absence of evidence to the contrary, it seems likely that our measurements of hydroxyproline in the excised culture areas provide mainly an index of fibril and fibre formation so frequently reported histologically. They in fact provide no guide as to the form of the diffused hydroxyproline material, and further experiments are necessary to solve this problem.

Another point of interest lies in the timing of hydroxyproline production. While a twofold increase occurred by the 10th day, this was not comparable with the eightfold increase recorded by GERADE and JONES[8] by the 12th day. They, however, were working with adult fibroblasts in roller tube culture. By 21 and 28 days there was in our experiments a significant increase over the 10-day figures, although no such difference existed between the 21- and 28-day results themselves. These findings agree well with those of HASS and MACDONALD[13] whose histological studies of fibroblast cultures showed collagen deposits at 6–8 days, increasing in amount until 14–16 days. They suggested, however, that after this time collagen began to be reabsorbed, and our experiments offer no confirmation of this possibility.

Comparison can be made between hydroxyproline formation in culture and collagen deposition in repairing wounds. ABERCROMBIE, FLINT, and JAMES[14] studied collagen formation in rat skin repairs up to 25 days after wounding. They found a significant difference between 10- and 25-day collagen levels, but although the mean collagen content of the wounded areas was greater at 25 than at 15 days, these means did not differ significantly. While it is certain (ABERCROMBIE and JAMES[15]) that collagen formation in wounds continues long beyond 25 days, the parallel with our present results is striking up to this time. In culture total hydroxyproline production at 10 days was significantly less than that at either 21 or 28 days, and while the 28-day mean exceeded that after 21 days culture, these means did not differ significantly.

This work has been supported by a grant of F. Hoffmann La Roche, Basle and we are grateful also to the Wellcome Foundation for providing one of us (D.W.J.) with a travel grant.

L. HULLIGER*, D. W. JAMES**, and M. ALLGÖWER***

Department of Surgery, University of Basel (Switzerland), 17. September 1957.

Zusammenfassung

Kulturen von Hühnerherzfibroblasten wurden nach 10, 21 und 28 Tagen *in vitro* auf ihren Gehalt an Hydroxyprolin – einer wichtigen Aminosäure des Kollagens – untersucht. Nach 10, bzw. 21 Tagen ist eine signifikante, durch Prolinzugaben nicht beeinflussbare Vermehrung des zu 50% löslichen Hydroxyprolins feststellbar.

[6] A. MAXIMOV, Zbl. allg. Path. path. Anat. *43*, 145 (1928).

[7] K. R. PORTER and P. VANAMEE, Proc. Soc. exp. Biol. N. Y. *71*, 513 (1949).

[8] H. W. GERARDE and M. JONES, J. biol. Chem. *201*, 553 (1953).

[9] D. W. JAMES, J. Path. Bact. *69*, 33 (1955).

[10] L. DOLJANSKI and F. ROULET, Virchow's Arch. *291*, 260 (1933).

[11] P. VANAMEE and K. R. PORTER, Fed. Proc. *10*, 263 (1951).

[12] S. F. JACKSON, Proc. Roy. Soc. [B] *142*, 536 (1954).

[13] G. HASS and F. MCDONALD, Amer. J. Path. *16*, 525 (1940).

[14] M. ABERCROMBIE, M. H. FLINT, and D. W. JAMES, J. Embryol. exp. Morph. *2*, 264 (1954).

[15] M. ABERCROMBIE and D. W. JAMES, J. Embryol. exp. Morph. *5*, 171 (1957).

* University of Florida, Gainsville.

** University College, London.

*** Kantonsspital, Chur.