THYMIDINE DEGRADATION PRODUCTS IN PLANT TISSUES LABELED WITH TRITIATED THYMIDINE

S. T. TAKATS, Ph.D., and R. M. S. SMELLIE, Ph.D.

From the Department of Biochemistry, The University of Glasgow, Scotland. Dr. Takats' present address is the Department of Biology, Temple University, Philadelphia, Pennsylvania

ABSTRACT

A study of the metabolic pathways of H^3-thymidine utilization in buds of *Lilium longiflorum* and root tips of *Vicia faba* was undertaken in order to obtain information that might explain the binding of H^3 from H^3-thymidine in the cytoplasm of these plants. H^3-thymidine was administered for various periods of time, the tissues were fixed and processed in the manner routinely used in preparation for sectioning and autoradiography, and the radioactivity removed in this way from the tissues was determined. It was found that the ethanol/acetic acid fixative contained the major portion of the radioactivity. Analysis of this extract by paper chromatography showed that the radioactivity was distributed among various degradation products of thymidine, principally β-ureidoisobutyric acid and β-aminoisobutyric acid. Time course experiments with *Vicia* showed that these degradation products rapidly appeared in the tissue during incubation with H^3-thymidine, while H^3-thymine appeared in the incubation medium. Preliminary studies indicated that *Vicia* root tips incubated with H^3-dihydrothymine for 24 hours would bind a small amount of H^3 non-specifically in the cells. It seems unlikely that utilization of degradation products of H^3-thymidine is sufficient to explain labeling which is concentrated in the cytoplasm.

One anomalous result in autoradiographic experiments using H^3-TdR[1] as a selective precursor of DNA has been the binding of label in the cytoplasm of the cells. Apart from cells known to be infected with DNA-containing virus and certain animal eggs in which there is chemical evidence of a cytoplasmic reserve of DNA (4), this has been difficult to interpret. Two cases (3, 19) have been interpreted as labeling of chloroplasts. In *Amoeba*, where it was originally reported (14), evidence has now been found for the existence of cytoplasmic particles containing DNA (16). In several other cases, however, (1, 2, 17, 20, 21, 23), the labeling has not been associated with any cytoplasmic structure. When H^3-TdR of very high specific activity is used, or especially after relatively long term administration of H^3-TdR, the cytoplasmic label has been shown to be partially resistant to extraction with hot acid or DNase (1, 13, 20, 21, 23), suggesting that it might be present in protein or other non-nucleic acid material.

The experiments reported in this communication explore some of the metabolic pathways by

[1] The following abbreviations are used in this paper: TdR, thymidine; TMP, thymidine-5′-monophosphate; TDP, thymidine-5′-diphosphate; TTP, thymidine-5′-triphosphate; DHT, dihydrothymine; DHU, dihydrouracil; BUIB, β-ureidoisobutyric acid; BAIB, β-aminoisobutyric acid; U, uracil; T, thymine; HMU, 5-hydroxymethyluracil; DNase, deoxyribonuclease I; PCA, perchloric acid; PDAB, *p*-dimethylaminobenzaldehyde.

which H^3-TdR may be utilized in buds of *Lilium longiflorum* and in roots of *Vicia faba*. Both these plants bind H^3 from TdR in the cytoplasm (20, 21, 23) and, in the absence of DNA synthesis in the nuclei, the label is concentrated in the cytoplasm. The present findings indicate that H^3-TdR is rapidly degraded during the time that the tissues are taking it up from the incubation medium. Similar results have been reported (6) for the metabolism of T in seedlings of rape. Since labeled degradation products of H^3-TdR could enter the citric acid cycle (15) and be bound by the cell in non-nucleic acid form, it was considered possible that this might account for the observed cytoplasmic labeling. A preliminary test of this possibility has shown that only a small amount of non-specific labeling of cells of *Vicia* root tips results from long term administration of H^3-DHT.

MATERIALS AND METHODS

Growing buds of *Lilium longiflorum* var. Croft were allowed to take up H^3-TdR (Schwarz BioResearch, Inc., Orangeburg, New York, 1.9 c/mM) in dilute Bonner's solution by the method described previously (22). The buds were fixed in fresh 3:1 absolute ethanol:glacial acetic acid for approximately 2 hours at 18–20°C, left overnight in 70 per cent ethanol at 4°C, rinsed in 70 per cent ethanol, dehydrated in 95 per cent and absolute ethanol, transferred successively to 3:1, 1:1, and 1:3 absolute ethanol:xylene and then pure xylene prior to infiltration with paraffin. Each of the extracts was retained. These procedures were carried out in order to duplicate those routinely used in the preparation of tissues for sectioning and autoradiography and to account for the radioactivity appearing in the extracts.

Seeds of *Vicia faba* var. Broad Windsor were surface-sterilized with $HgCl_2$, germinated in moist, autoclaved vermiculite for 8 to 10 days at 22 ± ½°C, and the seedlings transferred to continuously aerated, dilute Bonner's solution containing H^3-TdR (Schwarz BioResearch, 0.36 c/mM, or Radiochemical Centre, Amersham, Buckinghamshire, 4.4 c/mM). Terminal 3 to 5 mm segments of secondary roots were excised and fixed in fresh 3:1 absolute ethanol:glacial acetic acid for 1 hour at 18–20°C. They were then washed, dehydrated, and embedded in paraffin in the same way as described above for the *Lilium* buds. The extracts were retained for counting and chromatography.

Sections mounted on slides were extracted with boiling 3:1 absolute ethanol:ether for 5 minutes to remove lipids and with ice cold 5 per cent PCA for 15 minutes to remove acid-soluble materials. They were then treated with DNase (Sigma, crystalline) at a concentration of 0.1 mg/ml in deionized water, pH 5.5, containing 10^{-3} M Mg^{++}, for 2 hours at 37°C, or stained by the Feulgen reaction after 10 minutes' hydrolysis in N HCl at 60°C, or extracted with 5 per cent PCA in a boiling water bath for 15 minutes to remove all nucleic acids. Autoradiographs were made by applying Kodak AR.10 stripping film, drying, and exposing at 4°C with silica gel desiccant. The slides were developed in Kodak D19b for 6 minutes at 18°C, rinsed, fixed for 8 minutes in Kodak acid fixer diluted 1 part acid fixer to 2 parts distilled water, and finally washed for 2 hours in running tap water.

Chromatography was carried out on unwashed Whatman No. 1 chromatography paper with the solvent systems specified below. Marker compounds for purposes of chromatographic identification were obtained from the California Corporation for Biochemical Research, Los Angeles. BUIB was prepared by alkaline hydrolysis of DHT (8). Marker spots on the chromatograms were located by ultraviolet absorption or by spraying with ninhydrin or PDAB (8). The chromatogram strips were scanned for radioactivity using a Nuclear-Chicago 2 pi Actigraph system which at maximum sensitivity could detect approximately 1500 CPM (0.002 μc) H^3 in a spot 1 inch in diameter. Radioactivity of extracts and eluates of the chromatograms was determined by plating appropriately diluted aliquots on stainless steel planchets and counting these with a Nuclear-Chicago windowless gas flow counter.

H^3-DHT and H^3-BUIB were synthesized from H^3-T (Radiochemical Centre, Amersham, Buckinghamshire, 0.324 c/mM generally labeled) by hydrogenation in the presence of a rhodium catalyst according to the procedure of Green and Cohen (11). Approximately 70 per cent of the radioactivity initially in T was recovered in DHT. The H^3-DHT was separated from H^3-T by chromatographing twice in Fink's No. 8 solvent (7). Some of this H^3-DHT was then converted to H^3-BUIB by treatment with 0.1 N NaOH at 30°C for 30 minutes (11). The product was acidified to pH 3 with 0.1 N HCl, desalted by extracting four times with absolute ethanol/0.1 N HCl (18), and separated from any residual H^3-DHT and other unidentified radioactive products by chromatographing twice in Fink's No. 5 solvent. Since both H^3-T and H^3-TdR were labeled by the Radiochemical Centre in the same way (by an exchange reaction between $H_2{}^3O$ and T in the presence of a platinum catalyst), it is likely that the H^3-DHT and H^3-BUIB prepared from the H^3-T were labeled in the same position that they would have been had they been prepared from H^3-TdR. Their specific activities were not determined.

TABLE I

Total Radioactivity (Counts per Minute) in Extracts after Administration of H^3-TdR to Lilium Buds for 24 Hours

Extract	Bud 1, 14.0 mm (meiotic)	Bud 2, 14.0 mm (meiotic)	Bud 3, 14.0 mm (meiotic)	Bud 4, 9.5 mm (premeiotic)
Ethanol/acetic acid fixative	120,400	124,200	198,100	63,800
70 per cent ethanol (1st wash)	31,800	29,200	55,100	35,100
70 per cent ethanol (2nd wash)	2,900	3,190	8,220	1,590
95 per cent ethanol	0	0	750	0
Absolute ethanol	0	0	0	0
Absolute ethanol/xylene (combined)	0	0	0	0

TABLE II

Identification of Radioactivity in Ethanol/Acetic Acid Extracts of Lilium Buds after Administration of H^3-TdR for 24 Hours

Compound	R_f in solvent systems of Fink *et al.* (7)				
	No. 8	No. 2	No. 3	No. 5	No. 6
H^3-1	0.65	0.21*	—	0.87	0.05
H^3-2	0.05	0.37	0.74	—	—
TdR	0.26	—	—	—	—
T	0.50	—	0.70	—	—
DHT	0.64	0.82	—	0.59	0.52
DHU	0.40	—	—	—	—
BUIB	0.72	(Tailed)	—	0.78	0.05
BAIB	0.05	0.40	0.73	0.20	—
β-Alanine	0.02	0.27	0.60	—	—

* This value is based on duplicate runs, but was not repeatable. On subsequent chromatography with authentic BUIB, consistently bad tailing of BUIB resulted and radioactivity could not be localized in a spot.

RESULTS AND DISCUSSION

Four growing buds of *Lilium* were incubated for 24 hours in medium containing 20 μc H^3-TdR/ml (Schwarz, 1.9 c/mM). Three of the buds were at a late leptotene stage of meiosis and the fourth was premeiotic. After incubation, the buds were fixed, washed, and dehydrated as already described, and aliquots of each of the extracts were plated and the radioactivity determined. The results (Table I) showed that virtually all the soluble radioactivity in the tissue was extracted in the fixative and the first 70 per cent ethanol wash. Samples of the fixative from each experiment, containing approximately 20,000 CPM, were applied to chromatography paper and the papers were developed by descending chromatography in a solvent consisting of 100 ml isobutyric acid, 55.8 ml water, 4.2 ml ammonium hydroxide (sp gr 0.880), and 1.6 ml 0.1 M versene (12). The chromatograms were dried and scanned for radioactivity. The fixatives from each of the four buds showed two radioactive peaks, H^3-1 and H^3-2, the R_fs of which relative to TdR were 0.85 and 1.10, respectively. The areas corresponding to these peaks were eluted with 70 per cent ethanol or water, lyophilized, and rechromatographed with appropriate markers in several of the solvent systems of Fink *et al.* (7). The results, summarized in Table II, indicate that H^3-1 was H^3-BUIB and H^3-2 was H^3-BAIB. No trace of

radioactivity was detected in phosphorylated derivatives of TdR although the autoradiographs of the tissue showed typical labeling in acid-insoluble material of the nuclei and cytoplasm, and it was known that TMP, TDP, and TTP were stable in the fixative under the conditions of the experiment.

Additional experiments on H^3-TdR utilization were carried out with *Vicia*, since suitable *Lilium* material could no longer be obtained. In two initial experiments, plants were grown for 18 hours in medium containing 2 μc H^3-TdR/ml (Schwarz, 0.36 c/mM). The medium before and after incubation was checked for radiochemical purity by chromatography in Fink's No. 8 solvent and in the ammonium isobutyrate solvent, under conditions where it would have been possible to detect an impurity of 10 per cent or more. All the radioactivity in the medium before incubation was found in TdR. During the incubation, however, this disappeared and the chromatograms developed in these solvents showed only a tiace of radioactivity at the origin. As in the case of the *Lilium* buds, the fixative contained nearly all the soluble radioactivity of the tissue. The radioactive compounds in the fixative were resolved chromatographically in Fink's Nos. 8, 3, 6, and 1 solvents and in the 2-butanol:formic acid:water (75:15:10) solvent of Campbell (5). Four radioactive peaks appeared on scanning the chromatograms; three of these with R_fs of 0.0, 0.12, and 0.60 in Fink's No. 8 solvent were tentatively identified as H^3-β-alanine, H^3-BAIB, and H^3-BUIB, respectively; a fourth peak with an R_f of 0.91 in Fink's No. 8 solvent was present in larger amount than any of the other three but was not identified. Again, as in the *Lilium* buds, none of these compounds was on the pathway to DNA synthesis from TdR. The presence of H^3-β-alanine implied that the TdR was being degraded *via* the U to DHU pathway in addition to the T to DHT pathway and would suggest either that the H^3 in the administered TdR was not entirely on the methyl carbon of T (9) or that it was exchangeable from that position to the ring.

In these two experiments, the acid-insoluble radioactivity in the root tips made up approximately 25 and 40 per cent of the total radioactivity; of this, in both experiments, less than 5 per cent remained bound after extracting the nucleic acids with hot 5 per cent PCA. Autoradiographs of unextracted sections of these root tips showed a typical nuclear labeling pattern over the cells, which were dividing with a normal mitotic frequency. There was also an unexpected, heavily labeled surface material on the roots. This could not be identified and although a fungus was suspected neither hyphae nor nuclei could be seen. The surface label was resistant to extraction with hot 5 per cent PCA, to the N HCl hydrolysis used in the Feulgen reaction, and to DNase. The same material was found on the roots used in the time course experiments to be described below. Tentatively, it was considered to be either something adhering tightly to the roots from the vermiculite in which they had grown, or sloughed off root epidermis. Growth of seedlings in sand unfortunately was not satisfactory so that this obvious check on the source of the surface material could not be made.

The time course of conversion of H^3-TdR to degradation products was examined by sampling roots from separate plants after 1, 3, and 8 hours of continuous growth in medium containing 2 μc H^3-TdR/ml (Schwarz, 0.36 c/mM). Aliquots of the incubation medium were analyzed at each sampling time. The total radioactivity in the latter declined most rapidly during the first hour when the soluble radioactivity in the tissue rose. A striking change in the composition of the incubation medium also occurred: H^3-T replaced H^3-TdR. In one experiment a small amount of radioactivity was detected in T within 1 hour; in 3 hours the radioactivity was approximately equally distributed between T and TdR and in 8 hours no H^3-TdR was left (Fig. 1 *a*).

In the same experiment the fixative removed about 95 per cent of the soluble radioactivity from the tissue at each sampling time. Within 3 hours after the start of the incubation nearly all the radioactivity was present as H^3-BAIB (Fig. 1 *b*). The H^3-BAIB was distinguished from H^3-T, H^3-TdR, and H^3-β-alanine by chromatography in Fink's solvents Nos. 8 and 3.

Similar results were obtained in a second time course experiment using H^3-TdR from the Radiochemical Centre (2 μc/ml, specific activity 4.4 c/mM, hence about one-twelfth of the concentration of TdR used in the first experiment). In this case the replacement of H^3-TdR in the incubation medium by H^3-T was less rapid and after 8 hours the radioactivity was still distributed approximately equally between T and TdR. The fixative again showed a small initial amount of

radioactivity in TdR. This diminished with time and an increasing amount of radioactivity appeared in BAIB while no activity was detectable in TMP, TDP, or TTP. A repeat experiment with H^3-TdR from the Radiochemical Centre showed the presence of H^3-BUIB in the tissue after 1 and 3 hours' incubation but the major portion of the radioactivity was in BAIB.

Some of the roots in these three time course experiments did not bind any radioactivity in the cells at all. Since the chromatographic analysis of the fixative showed that H^3-BAIB was rapidly formed and since H^3-T was found in the incubation medium, it seems likely that the enzymes converting TdR to T and degrading the latter to BAIB may be active whether or not DNA synthesis is going on. This together with the additional possibility of degradation of formed DNA to BAIB by cell death (10) has yet to be tested adequately. It is unlikely that the peculiar radioactive surface material mentioned above represented the site of degradation of H^3-TdR since a few roots which were completely unlabeled, even lacking this surface material, after 1 hour still showed nearly complete redistribution of radioactivity from TdR to BAIB as well as a small amount of radioactivity in BUIB. The over-all results from the time course experiments with *Vicia* may be interpreted to mean that the roots rapidly degraded TdR to T, some of which passed out again into the medium while the remainder was converted to BAIB.

Only relatively small amounts of radioactivity appeared in compounds other than those mentioned above. In the media after 8 hours' incubation, one small peak of radioactivity was found at the origin of chromatograms developed in Fink's No. 8 solvent and this could be resolved into two peaks with R_fs of 0.0 and approximately 0.46 in Fink's No. 3 solvent. The latter was a broad peak lying between aspartic acid and β-alanine or between HMU and TMP when chromatographed with these markers. No further attempt was made to characterize it but on the basis of chromatographic behavior it was probably not a direct degradation product of either T or U. Occasionally a third very small peak of radioactivity with an R_f of 0.89 was observed when the medium from the 8 hours' incubation was chromatographed directly in Fink's No. 3 solvent. The fixative from roots incubated for 8 hours also showed traces of radioactivity which remained at the origin of chromatograms developed in the ammonium isobutyrate solvent.

The solution in which the roots had been grown prior to transferring them to the medium containing H^3-TdR was tested for its ability to degrade H^3-TdR, on the assumption that bacterial contamination could account for the formation of H^3-T in the incubation medium. The reaction was stopped with 3:1 absolute ethanol:glacial acetic acid after 1, 3, and 8 hours and aliquots were spotted directly on to paper for chromatography. The procedure was capable of detecting a radiochemical impurity of 7 per cent but no degradation of H^3-TdR was observed over the 8 hour period of incubation.

In order to make a direct autoradiographic test of the utilization of degradation products of TdR by *Vicia* root tips, H^3-DHT and H^3-BUIB were supplied in the incubation medium in place of H^3-TdR. A single time course experiment was run with each. *Vicia* seedlings were grown exactly as described for the experiments with H^3-TdR; one was transferred to medium containing 2 μc H^3-DHT/ml and the other to medium containing 0.375 μc H^3-BUIB/ml. Roots were excised from these single plants after 1, 3, 8, and 24 hours of continuous incubation and processed in the usual way. Autoradiographs of sections after 2 weeks' exposure showed appreciable labeling (three to four times background) only in the 24 hour roots of the H^3-DHT series. This label appeared to be distributed uniformly over the cells, not localized in any part, and was resistant to treatment with DNase and to the Feulgen hydrolysis. Thus it may possibly explain some of the non-specific labeling observed in experiments with H^3-TdR but does not account for labeling which, in the absence of DNA synthesis, is concentrated in the cytoplasm. No label was bound by the cells incubated with H^3-BUIB even after 24 hours, but this was probably due to the small amount of radioactivity in the incubation medium. Roots of both series showed the same heavy labeling of material on the edge of the roots as was observed in the experiments with H^3-TdR.

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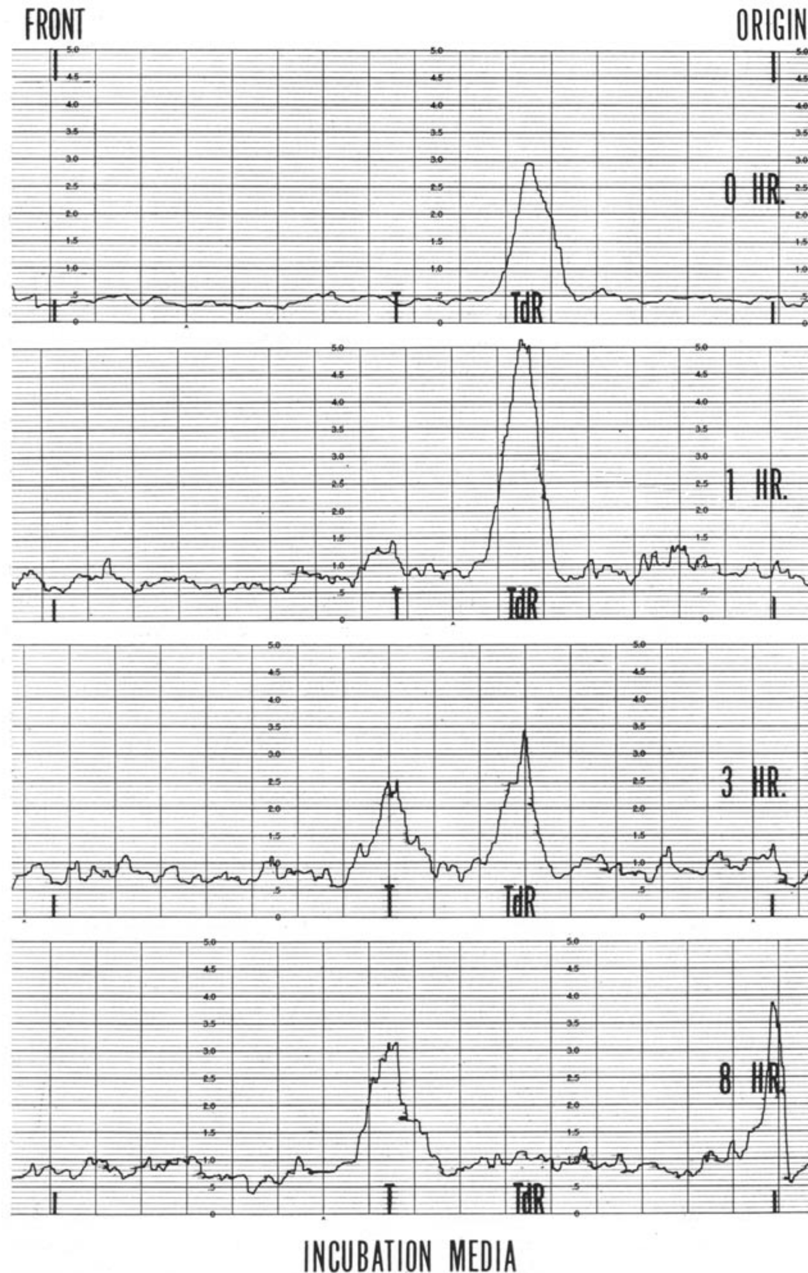

FIGURE 1a

FIGURE 1

Distribution of radioactivity at various times during growth of *Vicia* roots in solution containing 2 μc H^3-TdR/ml: (*a*) incubation media chromatographed in Fink's No. 8 solvent, and (*b*) fixatives chromatographed in isobutyric acid/water/ammonia/versene. Marker compounds included in the chromatograms are shown. All strips were scanned with a Nuclear-Chicago 2 pi Actigraph system; 6 to 10 per cent radiochemical impurity was detectable. The chromatogram of the 0 hour incubation medium was scanned at twice the count range of the others.

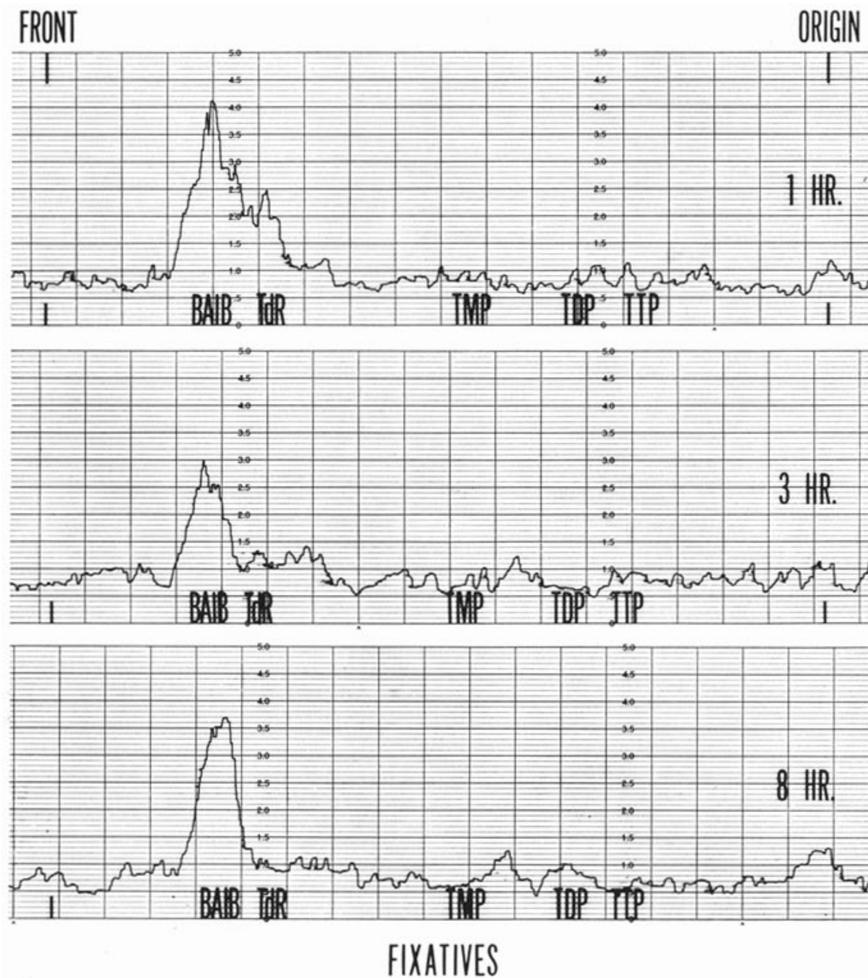

FIGURE 1*b*. *For legend see opposite page*

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Note added in proof: Two recent reports on cytoplasmic labeling following administration of H^3-TdR have come to our attention. Scher and Sagan (*J. Protozool.*, 1962, **9,** suppl., 13) have reported that *Euglena* will bind H^3-TdR in material that is removable with DNase and presumed to be in the chloroplasts. Lima-de-Faria (Abstracts, Second Annual Meeting of the Society for Cell Biology, 1962, 104) has found an intense, stable cytoplasmic labeling in the microsporocytes of the plant, *Agapanthus*. This label, as has been reported for other cases in the literature, is resistant to DNase.

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