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QUANTITATIVE TRITIUM EXCHANGE OF [^{3}H] AFLATOXIN B_1 DURING PENETRATION THROUGH ISOLATED HUMAN SKIN

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Tritium labelled aflatoxin B_1 ([G-^{3}H]AFB_1) underwent an almost total tritium exchange with water during penetration through isolated human skin. The process was not enzymatic and the site of exchange appeared to be within the epidermis. The mechanism which mediated this extensive exchange was not determined. However, the tritium in [G-^{3}H]AFB_1 was found to be very susceptible to chemical conditions which favored carbanion formation at the α-carbon of the cyclopentenone ring. The relative effectiveness of the various solvents in mediating the loss of the tritium label was 1 N NaOH >> methanol > 1 N HCl > water. This work serves as a warning that [G-^{3}H]AFB_1 can easily undergo significant changes in specific activity in biological tissues and under relatively mild experimental conditions. It is possible that conditions within the skin favor carbanion formation.

Tritium labelled aflatoxin B_1 ([^{3}H]AFB_1) is utilized in many different types of analytical studies where only a few molecules of AFB_1 are incorporated and thus high specific activity is required. The ease with which hydrogen exchanges with highly tritiated solvents or tritiated metal hydrides under moderate conditions makes high specific activity tritium-labelled compounds relatively inexpensive to produce, however, the exchange of tritium with environmental hydrogen can be a problem. Fortunately, this is not insurmountable if the extent of the exchange can be measured. For example, acid hydrolysis of [^{3}H]AFB_1-adducts, corrected for tritium exchange was used to obtain strong evidence that AFB_1-2,3-oxide was the probable ultimate carcinogenic metabolite of AFB_1 (1). It has been reported that in aqueous environments at 37°C [^{3}H]AFB_1 exchange occurs at a rate of 6% per day and at -80°C exchange is insignificant (2). To the best of our knowledge the

Abbreviations: AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; PBSA, Dulbecco's phosphate buffered saline with antibiotics.

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present report is the first to document an almost total tritium exchange utilizing [^{3}H]AFB$_1$ in a biological system and the first to demonstrate the near total exchange of a tritium labelled compound during penetration through human skin. This work serves as a warning that [G-^{3}H]AFB$_1$ can easily undergo significant changes in specific activity in biological tissues and under relatively mild experimental conditions.

MATERIALS AND METHODS

Chemicals and Test Agents. Aflatoxin B$_1$ was obtained from Calbiochem (LaJolla, CA, USA). Radiolabelled aflatoxins were obtained from Moravek Biochemicals Inc. (Brea, CA, USA): [G-^{3}H]AFB$_1$ (8 to 20 Ci/mmol, lots 1090, 2044, 11102), [G-^{14}C]AFB$_1$ (50 mCi/mmol,lot 5090), and [8,9-^{3}H]AFB$_2$ (60 Ci/mmol, lot 6025). Prior to utilizing tritiated compounds, the residues were dried under N_2 to drive off volatile tritiated contaminants. The radiochemical purity of [G-^{3}H]AFB$_1$ was >95% as determined by thin layer chromatography (TLC) on silica gel plates using chloroform-acetone-isopropanol (82.5:15:2.5) and toluene-acetone-ethyl acetate (55:15:30). The remaining radioactivity was generally distributed over the plates. The radiochemical purity of [8,9-^{3}H]AFB$_2$ was determined to be >98% based on High Performance Liquid Chromatography using a C18 reverse phase column and a mobile phase of 60% acetonitrile in water, 1 ml/min, UV detector at 362 nm and a Radiomatic Flo-One detector (Tampa, FLA). The radiochemical purity of [G-^{14}C]AFB$_1$ was as previously reported (3).

Test Systems. For the purposes of this communication, tritium exchange is defined as the amount of tritium radioactivity which partitioned into the aqueous phase following chloroform ($CHCl_3$) extraction of the various treatments to be described. Normally, an equal volume of deionize water and $CHCl_3$ were added to the various reaction mixtures and the aqueous phase was extracted 3 times with equal volumes of $CHCl_3$. The presence of AFB$_1$ in the $CHCl_3$ extracts was confirmed by a combination of TLC and High Performance Liqued Chromatography as described above. Also, samples containing sufficient quantities of AFB$_1$ were scanned on an AMINCO DW-2 UV spectrophotometer and the resulting UV spectra were compared to published spectra for AFB$_1$.

Tritium-exchange in phosphate buffered saline (PBSA), pH 7.1, with antibiotics was determined as described previously for measuring the stability of [3-^{3}H]T-2 toxin (4) except that 6 diffusion cells were assembled with either skin or Teflon^R disks (control) between the half chambers. Tritium exchange in a homogenate of viable epidermis was also determined. Briefly, the whole skin was heated to 60^{o}C for 1 min and then the epidermis was separated from the underlying dermis and homogenized in 50 ml of PBSA and then filtered through 3 layers of sterile cheese cloth. [G-^{3}H]AFB$_1$ was added to the homogenate to a final concentration of 11.4 nM. The homogenate plus AFB$_1$ was incubated in the dark, under N_2, with shaking for 20 hr at 22^{o}C. Samples were removed periodically and extracted with $CHCl_3$. Aliquots of both the $CHCl_3$ and aqueous phases were analyzed by liquid scintillation counting. A control with only PBSA was run concurrently.

Penetration of test agents through fresh whole skin (viable epidermis plus underlying dermis) or isolated epidermis from once frozen skin was conducted as previously described (3,5).

The relative stability of [G-^{3}H]AFB$_1$ was determined by measuring the change in specific activity which resulted from heating AFB$_1$ solutions in methanol, deionized water, 1 N HCL and 1 N NaOH for 15 min at 100^{o}C, under N_2 and in the dark. Following the heat treatment the AFB$_1$ solutions were extracted 3

times with $CHCl_3$ and the combined $CHCl_3$ extracts were dried over anhydrous Na_2SO_4 and then evaporated to dryness and the residues dissolved in $CHCl_3$. Aliquots of the $CHCl_3$ extracts were chromatogramed on silica gel TLC plates developed in $CHCl_3$-acetone-isopropanol. Aflatoxin B_1 was quantitated directly on the developed chromatograms by densitometric analysis (6) using an Aminco-Bowman Spectrophotofluorometer equipped with a Thin-Film Chromatogram Scanner. Radioactivity on developed plates was determined by scraping the areas corresponding to AFB_1 into vials and quantitating the radioactivity by liquid scintillation counting. The effects of the various treatments on the loss of the tritium molecule was determined by calculating the apparent specific activity of AFB_1 as the ratio of the counts per minute divided by the µg AFB_1 equivalents.

RESULTS AND DISCUSSION

In an earlier study (3) we demonstrated that [G-^{14}C]AFB_1 penetrates through isolated human epidermis very slowly and chemically unaltered. When we attempted to measure AFB_1 penetration using high specific activity [G-^{3}H]AFB_1 we found that 30 to 50 times as much ^{3}H penetrated as did ^{14}C (Fig. 1A). The [^{3}H]AFB_1 derived penetrant was water soluble and appeared to be ^3H_2O and the process by which this exchange occurred was not heat labile. Water soluble ^{3}H radioactivity accounted for 99% ± 20% (n=6) of the ^{3}H radioactivity recovered in the aqueous phase after $CHCl_3$ extraction of the

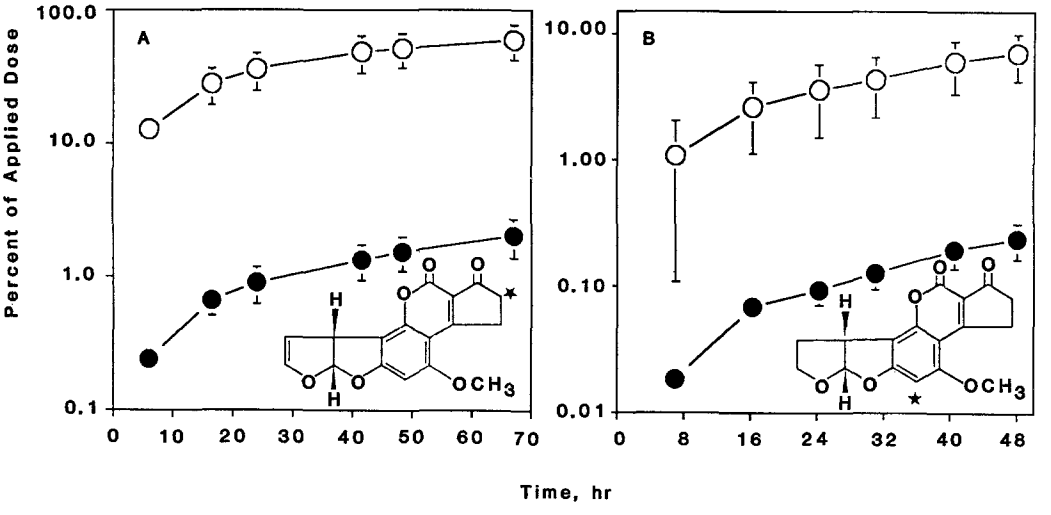

Figure 1. Activity-time curve for the appearance of radioactivity in the receptor fluid (PBSA) of diffusion cells. Data expressed as a percent of total radioactivity applied to epidermal surface of: A) epidermal disks cut from once frozen human skin and dosed by simultaneously applying 9.1 nmoles of AFB_1 in 20 µl of MeOH and containing both [G-^{3}H]AFB_1 (O—) and [G-^{14}C]AFB_1 (●—); and B) fresh whole skin (viable epidermis and underlying dermis) dosed by applying 0.44 nmoles in 30-40 µl of MeOH of either [G-^{3}H]AFB_1 (O—) or [8,9-^{3}H]AFB_2 (●—) . In all experiments the MeOH was allowed to evaporate and the surface of skin preparations were occluded by placing a glass coverslip over the donor chamber of each diffusion cell. Each point represents the mean plus and minus the standard deviation, n=6 to 12 replicates. The total recovery of the applied dose in A) was 116±6% (n=5) and 100±11% (n=6) for ^{14}C and ^{3}H, respectively. The total recovery in B) was 63% ± 14%, n=16. Inset are the structures of AFB_1 (A) and AFB_2 (B) showing the site of the tritium label (*) for [8,9-^{3}H]AFB_2 and the presumed site for [G-^{3}H]AFB_1 (1).

Table 1. Percent of ^{3}H in chloroform phase and aqueous phase of receptor fluid extracts 48 hr after the skin was dosed with [G-^{3}H]AFB_1 or [8,9-^{3}H]AFB_2 [a]

	Whole Skin	
	$CHCl_3$	Aqueous
[G-^{3}H]AFB_1	3% ± 3%[b]	97% ± 3%[b]
[8,9-^{3}H]AFB_2	18% ± 14%[c]	82% ± 14%[c]

[a] Data are means plus and minus the standard deviation, n=6. The rate of tritium exchange in PBSA was 6.1% per day for [G-^{3}H]AFB_1 (see Table 2). Based on this rate, the calculated percent of ^{3}H in the $CHCl_3$ and aqueous phases would be 83% and 17% respectively, after 48 hr in PBSA.

[b,c] Values with different superscripts differ significantly ($P < 0.05$); only columns were compared.

receptor fluid. Evaporation of the aqueous phase to dryness in a vacuum desiccator (23°C, 29 inches water, 1 hr) resulted in the volatilization of 99.3%± 0.2% (n=3) of the ^{3}H radioactivity. Fractional distillation of the pooled receptor fluids, after $CHCl_3$ extraction, resulted in 88% of the tritium being recovered at 99°C to 100°C. The results are consistent with the conclusion that the tritiated product was $^{3}H_2O$. Heating epidermal disks to 100°C (1-5 min) increased the penetration of both non-polar (extractable with $CHCl_3$) ^{3}H and ^{14}C radioactivity. However, 67% of the ^{3}H recovered in the receptor fluid was not extractable with $CHCl_3$ and volatilized under vacuum, while only 3% of the ^{14}C radioactivity was recovered as water soluble compounds.

Both generally labelled [G-^{3}H]AFB_1 and specifically labelled [8,9-^{3}H]AFB_2 (Fig. 1, insets) underwent tritium exchange during penetration through whole skin (Table 1). However, tritium penetration from [G-^{3}H]AFB_1 was 30 to 60 times greater than ^{3}H from [8,9-^{3}H]AFB_2 (Fig. 1B). Based on the results of our studies using [G-^{14}C]AFB_1 (3), we would expect aflatoxins to penetrate very slowly through isolated skin. The significantly slower accumulation of ^{3}H using specifically labelled [8,9-^{3}H]AFB_2 indicates that the generally labelled [G-^{3}H]AFB_1 is much more susceptible to tritium exchange and also suggests that the [8,9-^{3}H]AFB_2 could contain small amounts of tritium in an easily exchangeable position. Alternatively, there could be reductases which could mediate the exchange.

The site of tritium exchange between AFB_1 and water appears to be either within or on the epidermis and not within the receptor fluid. This conclusion is based on the fact that the lability of the tritium label in PBSA (Table 2) was not sufficient to explain the extent of tritium exchange between [G-^{3}H]AFB_1 and water during penetration through the isolated epidermal disks (Fig. 1A). When [G-^{14}C]AFB_1 was incubated under Teflon^{R} or epidermal disks there was no change in the percent $CHCL_3$ extractable

Table 2. Stability of [G-^{3}H]AFB_1 and [G-^{14}C]AFB_1 in PBSA [a]

	Teflon^R	Epidermal Disks	Control
[^{14}C]AFB_1	94.9 ± 0.6[b]	95.0 ± 1.0[b]	97.0 ± 2.6[b]
[G-^{3}H]AFB_1	90.8 ± 1.7[c]	88.3 ± 2.4[c]	96.0 ± 1.1[b]

[a] Values are percent of radioactivity extracted into $CHCl_3$ from PBSA after 24 hr in diffusion cells. Data are means plus and minus the standard deviations, n=3. The cells were assembled with either Teflon^R or epidermal disks separating the half-chambers and radiolabelled AFB_1 was added directly to the PBSA in the receptor chambers. The controls were solutions of radiolabelled AFB_1 in PBSA which were extracted with $CHCL_3$ immediately after solubilizing AFB_1 in the buffer.

[b,c] Values with different superscripts differ significantly ($P < 0.05$).

radioactivity relative to controls. However, with [G-^{3}H]AFB_1 there was a small but statistically significant decrease in the percent $CHCl_3$ extractable radioactivity. This decrease was evident with either Teflon^R or skin mounted between the half cells and thus can not be attributed to exchange mediated by the epidermal disks. The rate of ^{3}H exchange in PBSA was 0.25% / hr ± 0.39% (n=6), a value similar to that found by Olsen et al.(2). For comparison, the rate of exchange from a surface deposit of [G-^{3}H]AFB_1 under water saturated aerobic conditions was 0.19% / hr± 0.02% (n=3). The calculated rate of tritium exchange during penetration through epidermal disks (from Fig. 1A) was 2.1% /hr, 1.5% /hr, and 1.04% /hr, at 6 hr, 24 hr, and 48 hr, respectively. Chloroform extraction of the receptor fluid and skin from the penetration experiment illustrated by Fig. 1A revealed that 94% ± 1% (n=6) of the tritium label on/or within the epidermal disks was present as $CHCl_3$ extractable compounds whereas in the receptor fluid only 0.3% ± 0.3% of the tritium label was $CHCl_3$ extractable. This finding is consistent with the conclusion that the exchange process occurs within the epidermis. Alternatively, tritium exchange could occur on the skin surface with the resulting ^3H_2O penetrating rapidly and preferentially relative to [^{3}H]AFB_1. The rate of tritium penetration from [G-^{3}H]AFB_1 through whole skin (from Fig. 1B) was 0.15% /hr and exhibited first order kinetics over the entire 48 hr period. The rate of AFB_1 tritium exchange as a surface deposit (0.19% /hr) can account for the ^{3}H which accumulated in the receptor fluid (Fig. 1B) if all of the ^3H_2O formed on the surface penetrated through the whole skin. However, the stability of [G-^{3}H]AFB_1 in either PBSA or as a surface deposit, cannot account for the rate of tritium exchange observed with epidermal disks (Fig. 1A).

The quantitative difference between once frozen epidermal disks and fresh whole skin may reflect differences in barrier properties resulting from the procedures involved in the processing and storage of the epidermal disks. It is well documented that freezing can affect both the permeability properties of the stratum corneum and the viability of the underlying viable epidermis (5,7). In an earlier study we observed extensive exchange (84%)

Table 3. Change in specific activity of [G-^{3}H]AFB_1 after heating in various solutions at 100°C for 15 min, under N_2 and in the dark

Solution	Apparent Specific Activity[a]
Methanol (not heated)	1.74 ± 0.59
Methanol	0.35 ± 0.10
Deionized water	1.10 ± 0.19
HCl (1N)[b]	0.60 ± 0.04
NaOH (1N)[c]	0.05 ± 0.01

[a] Values are cpm x 10^6 / µg AFB_1 equivalents. Samples of the $CHCL_3$ extracts were diluted in MeOH and scanned on an AMINCO DW-2 UV spectrophotometer to verify the presence of aflatoxin. Data are means plus and minus the standard deviations, n=4.

[b] Treatment with HCl converted all of the AFB_1 to an aflatoxin derivative which had a mobility on the silica gel thin layer plate of approximately 0.5 relative to AFB_1. It was assumed that this compound was a hemiacetal derivative formed by acid-catalyzed hydration (10).

[c] The NaOH treated AFB_1 solution was acidified before extraction with $CHCl_3$.

using fresh epidermal disks obtained from weanling pigs. However, only 2.5% of the applied dose had penetrated the fresh isolated epidermal disks after 48 hr.

If exchange occurs within the epidermal disks then the process appears to be non-enzymatic. While there are hydrolytic enzymes which can catalyze tritium exchange with water prior to hydrolysis of the substrate, it is unlikely that the tritium exchange we observed is enzymatic for two reasons. First, the exchange between [G-^{3}H]AFB_1 and water was not heat labile, and second, in our studies with [G-^{14}C]AFB_1 (3) we found no evidence of significant metabolism of AFB_1 during penetration through epidermal disks from once frozen human skin. We compared tritium exchange in PBSA and the homogenate of isolated epidermis and found that the rate of tritium exchange was 0.36% / hr ± 0.04% and 0.47% / hr ± 0.04%, respectively. While statistically significant ($P < 0.05$), the difference of 0.1% / hr would not be sufficient to account for the extensive exchange observed in the penetration experiment using epidermal disks (Fig. 1A). In a separate experiment it was found that heating the homogenates to 100°C and incubation under air had no effect on the amount of exchange.

We have studied the cutaneous penetration and metabolism of tritiated diacetoxyscirpenol, T-2 toxin, and verrucarin A and have not observed extensive tritium exchange (8). Only [^{3}H]AFB_1 and, to a much lesser extent, [^{3}H]AFB_2 have undergone extensive exchange. Because the preparation of tritiated AFB_1 requires relatively mild conditions (9) the loss of tritium from [G-^{3}H]AFB_1 should always be considered when designing studies. It has been suggested that most of the ^{3}H in [G-^{3}H]AFB_1 is on the α-carbon of the

cyclopentenone ring (1) (Fig. 1, inset). The hydrogen at this position can be easily exchanged with water via conditions which favor carbanion formation (Table 3). The fact that the tritium molecule is most susceptible to exchange in dilute base and much less susceptible in dilute acid (Table 3), is in agreement with the findings of Swenson et al. (1) that most of the tritium in [G-^{3}H]AFB$_1$ is located on the α-carbon of the cyclopentenone ring. Treatment of AFB$_1$ with weak base and even refluxing in water are known to open the lactone ring (10, 11), however, rather than losing hydrogen, opening of the lactone ring results in addition of hydrogen (substituted o-coumaric acid) which is subsequently lost if the ring structure is closed by acidification. Additionally, treatment of ketones with NaOH results in the loss of one of the hydrogens on the α-carbon (carbanion formation). It is possible that the chemical environment through which AFB$_1$ passes when penetrating the epidermis favors carbanion formation and thus facilitates the extensive loss of ^{3}H from [G-^{3}H]AFB$_1$.

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