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Non-human Primate Platelets and Arterial Tissue Cannot Convert Preformed [^{14}C]Lanosterol into [^{14}C]Cholesterol *in vivo*

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In contrast with the prevailing view, we report the inability of non-human primate platelets or arterial tissue to complete the biosynthesis of [^{14}C]cholesterol from [^{14}C]mevalonic acid *in vitro* or *in vivo*, or from preformed [^{14}C]lanosterol *in vivo*. The latter observation suggests that these tissues lack one or more components of the methyl sterol demethylase system.

In 1973 (Derksen & Cohen) we reported that human platelets can synthesize lanosterol and dihydrolanosterol from mevalonate, and cholesterol from desmosterol *in vitro*, but are unable to convert lanosterol into cholesterol. We also found that human coronary artery and aorta are apparently incapable of carrying the label from mevalonate beyond lanosterol (Derksen & Cohen, 1973). The latter observation conflicted with previous reports describing cholesterol biosynthesis by arterial tissue (Azarnoff, 1958; Chobanian, 1968; St. Clair *et al.*, 1968; Daly, 1971), possibly owing to differences in technique. To resolve this important issue, we devised a unique experimental approach to determine whether arteries (or platelets) from non-human primates can convert preformed [^{14}C]lanosterol into [^{14}C]cholesterol *in vivo*.

Experimental

We adapted our techniques for human platelets *in vitro* (Derksen & Cohen, 1973) to rhesus-monkey platelets. Then an experiment was done *in vitro/in vivo* as follows. A no.-18-gauge silastic rubber tubing (Dow Corning, Midland, MI, U.S.A.) fitted with a Luerstub adapter was placed in the right atrium of a 5 kg adult male rhesus monkey via the right jugular vein. A sample (80ml) of blood was withdrawn into a plastic syringe and transferred to a 300ml plastic pack (4R-20-11; Fenwal, Deerfield, IL, U.S.A.) for preparation of a platelet suspension. The latter was incubated for 1h with 15μCi of DL-[2-^{14}C]mevalonic acid lactone (7.1mCi/mmol; Amersham–Searle, Chicago, IL, U.S.A.) in 1ml of a buffered reaction mixture (Derksen & Cohen, 1973) and washed twice with 100ml of fresh buffer before re-infusion in a femoral vein. At 2h after infusion, a 50ml blood sample was drawn, via the right atrial catheter, for preparation of a platelet pellet that, in parallel with a portion of post-incubation platelets, was assayed as previously described (Derksen & Cohen, 1973).

Next, studies were done with arterial segments of two baboons (*Papio anubis*), a 10kg female and a 30kg male, and seven rhesus monkeys (*Macaca mulatta*, 4–6kg, two of which were male. All were starved for 12h before study, pre-medicated with Ketamine hydrochloride (10ml/kg intramuscularly), and maintained on 1% fluothane anaesthesia. There were three types of experiments with arterial segments: *in vitro*, *in vitro/in vivo* and *in vivo*.

The studies *in vitro* involved the male baboon and three rhesus monkeys, one of which was female. The aorta below the renal arteries, and the common iliac arteries, were excised and cut into 2cm segments, which were rinsed free of blood with 0.9% NaCl before being added, as intact tubes, to 2ml of a reaction mixture containing 5μCi of [2-^{14}C]mevalonic acid. After incubation at 37°C for 3h the segments were minced and homogenized in ice-cold 0.9% NaCl before protein determination and lipid analysis (Derksen & Cohen, 1973).

The studies *in vitro/in vivo* involved three female rhesus monkeys. A lower abdominal midline incision and a short incision overlying the left thigh were made. The abdominal aorta and its branches were exposed. Bulldog clamps were applied to the proximal end of the external iliac artery and distal end of the common femoral artery. A 2cm segment of iliac artery was excised between the clamps and immediately added to 1ml of the reaction mixture containing 15μCi of [2-^{14}C]mevalonic acid. After 45min incubation at 37°C the radioactive medium was decanted and the vessel washed twice in 50ml of fresh buffer before re-anastomosis at the site of removal to restore normal blood flow. The abdominal wound was then closed for 1h, after which it was re-opened and the involved arterial segment, still pulsating, was removed for analysis. At this time 2cm segments of

artery above and below the removed segment were taken for control studies *in vitro*.

The studies *in vivo* followed removal of the re-anastomosed arterial segment. Bulldog clamps were placed across the proximal end of the opposite common iliac artery and the aorta at the level of the renal arteries. A purse-string suture was placed in the anterior aspect of the ballooned aorta, through which the blood was evacuated via a no.-25-gauge needle. The same needle was used to wash the aortic segment several times with 0.9% NaCl before injection of 1ml of the reaction mixture containing 15μCi of [2-^{14}C]mevalonic acid. The purse-string suture was tied, and the abdominal wound closed for 1h. The wound was then re-opened and the clamps were removed to restore blood flow through the previously ballooned segment for 5min before removal of the segment for analysis.

Timed studies *in vivo* were done with one rhesus monkey and one baboon, both females. In each of these studies, 2cm segments of the left and right common and external iliac arteries (a total of four sites) were injected with 15μCi of [2-^{14}C]mevalonic acid as described above. After incubation for 1h, segments on the left side were emptied of their reaction mixtures by aspiration, rinsing and re-aspiration, after which the distal segment was excised for analysis (Derksen & Cohen, 1973), whereas the proximal segment was opened to the normal flow of blood for 1h before being removed for analysis. The same procedure was repeated with the segments on the right after a 2h incubation.

Results and Discussion

On a per mg of protein basis the total commitment of mevalonate to the sterol pathway was 4.2 times greater in platelets than in arteries; however, the distribution of radioactivity among identified compounds was similar, squalene and lanosterol accounting for 71 and 83% of the total incorporation by platelets and arterial tissue respectively. Only squalene and lanosterol remained after labelled platelets *in vitro* had circulated for 2h *in vivo*.

Plate 1 is a radioautogram of a representative study among the three in which experiments *in vitro*, *in vitro/in vivo* and *in vivo*, on arterial segments were carried out in the same animal. Most importantly, in all of these studies, labelling beyond lanosterol was conspicuously absent.

In the study *in vitro/in vivo* (Plate 1*a*), the ^{14}C label was found primarily with squalene and lanosterol, as in the analogous platelet experiment. This implies that for both arterial tissue and platelets preformed [^{14}C]farnesol and [^{14}C]farnesoic acid were metabolized or exchanged *in vivo*.

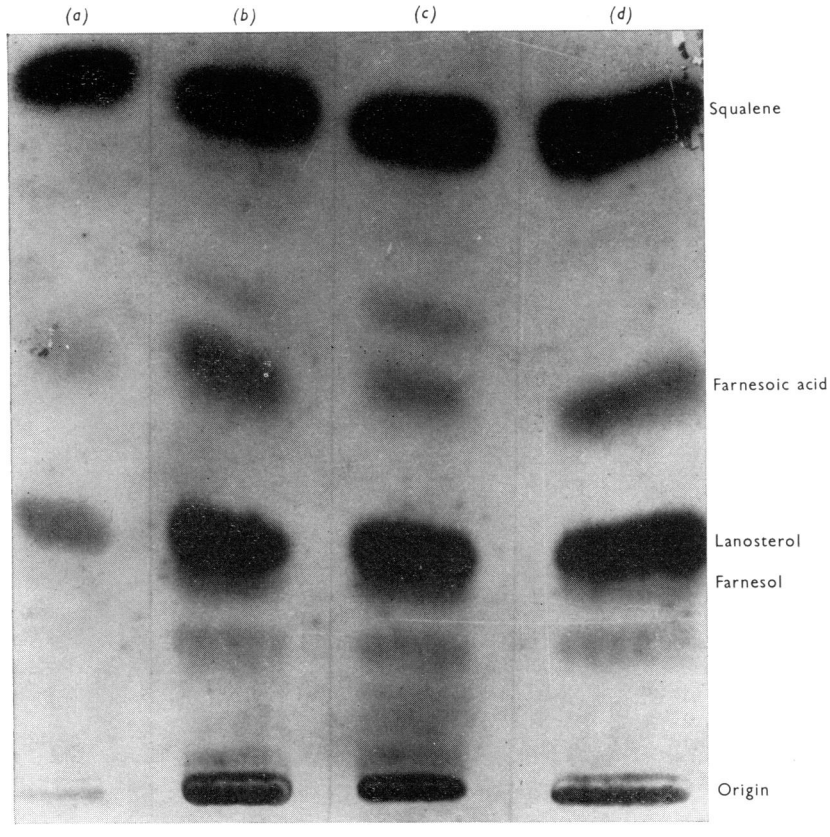
The studies *in vivo* (Plates 1*b* and 1*c*) and *in vivo* (Plate 1*d*) gave similar patterns of incorporation; both showed labelling of farnesol, farnesoic acid, squalene and lanosterol, as well as an unidentified compound that did not co-chromatograph in a system of argentation chromatography (Derksen & Cohen, 1973) with commercially available sterols. The total incorporation of [2-^{14}C]mevalonic acid into the sterol pathway *in vivo* was twice that of fresh arterial segments *in vitro*.

The latter finding was further elucidated by the timed studies *in vivo*. For the segments in which blood flow was never restored, the total incorporation of [2-^{14}C]mevalonic acid and its distribution among cholesterol precursors were approximately the same in the 1h and 2h samples (Table 1). Further, where arterial flow was restored for 1 or 2h after pulse-labelling with [2-^{14}C]mevalonic acid, there were decreases of 52 and 81% respectively in the total recovery of incorporated ^{14}C label. These results confirm the earlier assertion that cholesterol precursors were exchanged or metabolized *in vivo*. The labelled cholesterol precursors were not depleted at the same rate. After restoration of blood flow for 1h, the decreases in lanosterol (7%) and farnesol (38%) were much less than in squalene (77%) and farnesoic acid (67%). However, after restoration of blood flow for 2h, the decreases in farnesol (77%)

Table 1. *Fate of cholesterol precursors in primate arteries after pulse-labelling in vivo with* [2-^{14}C]*mevalonic acid*

Segments of iliac artery were incubated as described in the Experimental section under time-study *in vivo*. After homogenization, arteries (10mg of protein equivalents) were analysed as previously described (Derksen & Cohen, 1973). The results are expressed in c.p.m. and are the means of two studies, one in a rhesus monkey, the other in a baboon.

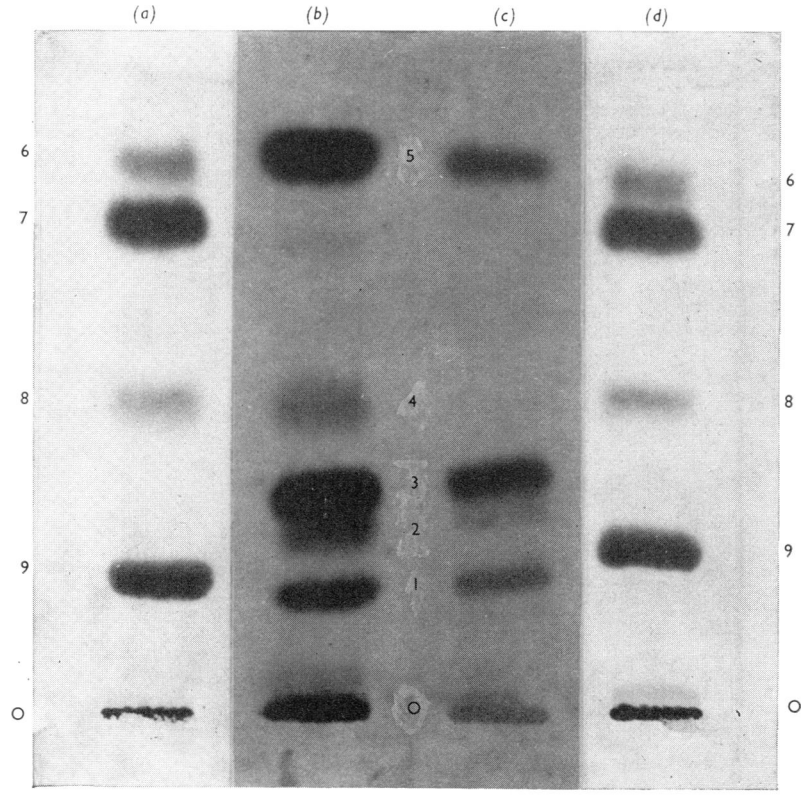
Time of labelling *in vivo* (h)	Time of blood flow after labelling (h)	Radioactivity incorporated (c.p.m.)						
		Farnesol	Farnesoic acid	Squalene	Other non-sterol	Lanosterol	Cholesterol	Total
1	0	1600	1200	14000	1700	8300	<75	26800
1	1	1000	400	3400	1200	7800	<75	13800
2	0	1800	1000	11000	2200	8800	<75	24800
2	2	400	100	1400	600	2200	<75	4700



EXPLANATION OF PLATE 1

Incorporation of [^{14}C]*mevalonic acid into the sterol pathway of non-human primate arteries*

Arterial segments (10mg of protein equivalents) were incubated with [^{14}C]mevalonic acid *in vitro* or *in vivo* as described in the Experimental section. After incubation, the arterial lipids were extracted and chromatographed in 0.5mm-thick silica-gel H with hexane/diethyl ether/18M-acetic acid (55:45:1, by vol.) as the developing system. (*a*) *In vitro*/*in vivo*. After incubation *in vitro*, a segment of artery was re-anastomosed and allowed to complete the incubation *in vivo*. (*b*) and (*c*) *In vitro*. After completion of (*a*), segments of artery from above (*b*) and below (*c*) the re-anastomosed segment were taken for control incubations *in vitro*. (*d*) *In vivo*. After completion of (*a*), (*b*) and (*c*), a clamped-off arterial segment was incubated *in vivo* after injection of [2-^{14}C]mevalonic acid into its lumen. In no case did the ^{14}C label progress beyond lanosterol.



EXPLANATION OF PLATE 2

Timed studies of sterol biosynthesis by non-human primate arteries in vivo

Lipid extracts from fresh arterial segments (10mg of protein equivalents) (*a* and *d*) were chromatographed alongside extracts from segments that had been incubated with [^{14}C]mevalonic acid *in vivo* for 2h with (*c*) and without (*b*) restoration of the blood flow for 2h. After radioautography, the bands of tracks (*b*) and (*c*) were scraped for radioactivity counting in a liquid-scintillation spectrometer (see Table 1), before spraying the silica with H_2SO_4 in preparation for charring. The radioautogram was fitted into its correct position between the charred tracks as shown in the photograph. The numbers at the sides refer to the bands that were visible on the silica after charring. The numbers in the centre are adjacent to the bands that were visible on the radioautograms: 1, non-sterol unknown; 2, farnesol; 3, lanosterol; 4, farnesoic acid; 5, squalene; 6, cholesterol esters; 7, triglycerides; 8, fatty acids; 9, cholesterol. Mevalonic acid and phospholipids remain on the origin (O) in this system. There was no overlap whatever between the cholesterol band and the adjacent radioactivity bands. Radioautograms showed no darkening over the cholesterol band even after 2 months' exposure of the film.

and lanosterol (75%) were in the range found with farnesoic acid (90%) and squalene (87%).

Most importantly, nearly 8000c.p.m. of [^{14}C]lanosterol was retained for 1 or 2h in an arterial segment under conditions that approached the natural state of circulation, yet less than 75c.p.m. was recovered in the cholesterol band (Table 1). It should be emphasized that these incubations were done in a buffered medium that was not in communication with the circulation, thus precluding loss of labelled cholesterol by exchange with erythrocyte or plasma pools. That cholesterol was not labelled is clearly shown in Plate 2, where there is no overlap whatsoever between the cholesterol band and adjacent radioactivity bands.

In summary, we believe that the experiments using incubations *in vivo* provided the platelets and arteries with a more favourable milieu for sterol biosynthesis than the incubation buffers used as a routine, and should have allowed cholesterol biosynthesis to proceed to completion. The fact that neither platelets nor arteries were able to synthesize [^{14}C]cholesterol from preformed [^{14}C]lanosterol in the studies *in vitro*/*in vivo* or from [^{14}C]mevalonate in the studies *in vivo* suggests that both of these tissues lack one or more components of the methyl sterol demethylase system. We believe that, in earlier investigations describing cholesterol biosynthesis by arterial tissue, another ^{14}C-labelled compound could have been responsible for the radioactivity that was attributed to cholesterol. Our methodology has allowed us to prove more definitively the presence of [^{14}C]farnesol and lanosterol as well as the absence of [^{14}C]cholesterol (Derksen & Cohen, 1973) (Plate 2).

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