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Enhancement of inflammation and histamine formation by actinomycin D

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1. An inflammation model developed in other laboratories was used in this study. Mice given endotoxin intranasally develop lung inflammation which progresses in intensity for several days. Lung weight is a satisfactory measure of inflammation.

2. In lungs of mice treated intranasally with endotoxin, histidine decarboxylase was activated within 6 hr, before lung weight had increased substantially. Enzyme activity was near maximal at 24 hr but had dropped by 48 hr, at which time inflammation was increasing. The data are consistent with an early role for histamine in mediating inflammation, but not with an essential role in the later stages.

3. When actinomycin D, an inhibitor of RNA synthesis, was mixed with endotoxin solution and given to mice intranasally, both lung inflammation and histidine decarboxylase activation were markedly enhanced at 24 and 48 hr, relative to effects produced by endotoxin alone.

4. Evidence is presented that intranasal instillation of endotoxin into mice increases histamine formation in lung *in vivo*.

5. We previously found protein synthesis inhibitors, the only drugs shown capable of blocking histidine decarboxylase activation, to be powerful anti-inflammatory agents. Now we have found that actinomycin D, the only drug shown capable of enhancing histidine decarboxylase activation, to be strongly pro-inflammatory. These observations support a causative role for histamine in slowly-developing inflammation, and also provide a rigorous test for the participation of other mediator candidates.

We have shown that several inhibitors of protein synthesis (cycloheximide, puromycin and tenuazonic acid) blocked activation of histidine decarboxylase induced by endotoxin given intraperitoneally; however, actinomycin D, an inhibitor of RNA synthesis, failed to block activation. Cycloheximide, in systemic doses which blocked histidine decarboxylase activation, also strongly suppressed inflammation of rat paw induced by local turpentine or carrageenin. In contrast, systemic actinomycin was devoid of anti-inflammatory effect (Schayer & Reilly, 1968).

We now show that actinomycin D, when added to endotoxin solution, and the mixture instilled into the nostrils of mice, can cause a marked enhancement of

lung histidine decarboxylase activation and of lung inflammation. We also report evidence suggesting that the increased histidine decarboxylase levels in lungs of mice given endotoxin intranasally, reflect an increased rate of histamine formation in lung *in vivo*.

Methods

Female albino CF-1 or CF-1 (S) mice, about 18–21 g, were obtained from Carworth, Inc., New City, New York. (^{14}C)-L-histidine, specific activity 57·8 mc/m-mole, was purchased from Nuclear-Chicago ; it was purified before use to remove traces of (^{14}C)-histamine (Schayer, 1968). (^{14}C)-histamine, specific activity 54 mc/m-mole, was from Nuclear-Chicago. Actinomycin D was generously provided by Dr. Clement Stone of The Merck Institute for Therapeutic Research. Cycloheximide (Actidione) was purchased from Nutritional Biochemicals, Inc. The endotoxin was Difco *Escherichia coli* lipopolysaccharide.

The method of Herrmann, Engle & Perlman (1959) was used to measure mouse lung inflammation. These authors, and others (Groupé, Dougherty & Manaker, 1956 ; Ginsberg, 1955) have shown that instillation of endotoxins or other irritants into nostrils of mice leads to a gradual development of lung inflammation, that the increase in lung weight is a satisfactory measure of inflammation, and that anti-inflammatory drugs can reduce the irritant-induced increase in lung weight.

Mice were anaesthetized with pentobarbitone ; then 0·05 ml. of a saline solution containing endotoxin, actinomycin D, or both, was instilled into the nostrils. Groups of mice were killed at various intervals, chilled, lungs removed, weighed and assayed for histidine decarboxylase activity by an isotope dilution method described in full by Schayer (1968a).

Three pooled lungs were homogenized with 3 ml. phosphate buffer, pH 7·4, containing glucose, centrifuged, and an aliquot incubated for 3 hr with pyridoxal phosphate and tracer amounts of (^{14}C)-L-histidine under nitrogen. The newly formed (^{14}C)-histamine was diluted with carrier, isolated and converted to benzenesulphonylhistamine (BSH). After recrystallization, BSH was weighed and counted in a Packard Tri-Carb liquid scintillation spectrometer, background about 20 c.p.m. ; at least 4,000 counts were obtained for each sample. Units of histidine decarboxylase are reported as d.p.m. (^{14}C)-histamine formed under our standardized conditions, for the entire tissue sample.

For the study on histamine formation *in vivo* (experiment 6) groups of mice were injected intravenously with 10 μc (^{14}C)-L-histidine per mouse and killed 4 min later. Tissues were homogenized in acidic buffer, and carrier histamine added to one aliquot. A second aliquot was incubated with a powerful specific bacterial histidine decarboxylase which converts free L-histidine to histamine quantitatively ; then carrier histamine was added. Histamine was extracted from both aliquots, then BSH prepared and counted as described above. Full details of the method have been published (Reilly & Schayer, 1968a). In experiment 7, mice were injected intravenously with (^{14}C)-histamine, 0·50 μc per mouse ; tissues were homogenized in 0·4 M perchloric acid and assayed for total (^{14}C) and for unchanged (^{14}C)-histamine as described above.

Results

Experiments 1–3 : effect of local actinomycin D on endotoxin-induced inflammation of mouse lung

Several preliminary experiments were performed to establish conditions for obtaining definite lung inflammation in mice. Satisfactory dosages were 25 μg endotoxin, 2·5 μg actinomycin D, or both, in 0·05 ml. saline, instilled into the nostrils.

Two aspects of the experimental procedure and treatment of data in experiments 1–3 require explanation.

(1) Lungs of mice treated intranasally with endotoxin, or endotoxin plus actinomycin, show considerable variation in inflammatory response presumably because variable amounts of irritant solution contact lung tissue. As our purpose was to relate histidine decarboxylase activity to inflammation it was considered necessary to eliminate lungs inadequately exposed to the test drugs. We therefore arbitrarily

TABLE 1. *Comparison of weight and histidine decarboxylase activity of lungs from mice treated intranasally with endotoxin, actinomycin D, or endotoxin plus actinomycin D*

	Intranasal treatment							
	Saline		Actinomycin		Endotoxin		Endotoxin+ actinomycin	
Time*	Wt.†	HD‡	Wt.	HD	Wt.	HD	Wt.	HD
Exp. 1, 24 hr	0·75	726			1·03	4,650	1·02	4,230
	0·82	332			1·09	2,950	1·22	10,800
	0·82	566			1·18	4,530	1·31	14,800
	0·90	753			1·18	11,900	1·35	18,800
	0·94	501			1·24	7,770	1·51	20,500
							2·08	31,600
Exp. 1, 48 hr					1·21	2,000	1·60	16,900
					1·21	1,230	1·68	19,000
					1·23	3,220	2·13	21,400
					1·43	4,390	2·80	21,600
Exp. 2, 6 hr	0·83	410	0·86	510	0·91	2,890	0·98	3,180
	0·87	640	0·87	470	0·94	4,700	1·00	3,480
	0·89	520	0·89	630	0·98	4,540	1·03	2,600
	0·91	430	0·91	410	1·01	4,890	1·06	2,170
	0·96	1,190	0·96	320	1·05	3,910	1·10	3,690
Exp. 3, 24 hr	0·77	580	0·93	1,260	1·12	3,900	1·19	5,790
	0·81	540	0·94	2,400	1·14	6,810	1·25	5,580
	0·84	540	0·98	1,690	1·18	4,930	1·31	6,550
	0·86	700	1·03	1,430	1·21	5,200	1·41	7,410
	0·97	1,670	1·14	1,300	1·29	5,350	1·55	15,900
Exp. 3, 48 hr	0·79	420	0·90	1,280	1·17	2,120	1·55	11,200
	0·82	450	0·96	1,190	1·22	2,230	1·61	13,900
	0·86	430	1·03	1,750	1·24	2,080	1·78	15,000
	0·90	510	1·12	2,760	1·34	3,040	1·96	23,600
	0·95	670	1·20	3,160	1·54	4,390	2·20	21,500

* Time from intranasal instillation to death.
† Mean weight of three pooled lungs (expressed as % body weight); lungs of comparable weight selected for pooling.
‡ Corresponding histidine decarboxylase activity units.
Experimental: Saline, endotoxin 25 μg, actinomycin 2·5 μg, or endotoxin 25 μg plus actinomycin 2·5 μg instilled intranasally in mice; groups of mice killed 6, 24 or 48 hr later.

discarded several lungs of lowest weight (% body weight), usually five, in each group.

(2) Histidine decarboxylase activities are reported as d.p.m. (^{14}C)-histamine formed, on a total tissue basis rather than a weight basis, because we believe that the number of cells in which enzyme activation may occur is not changed during inflammation (Schayer, 1962). Because of the large variability of data in some groups, only obvious differences are used in the interpretation.

Experimental conditions and results of experiments 1–3 are shown in Table 1.

Experiment 4 : effect of local cycloheximide on endotoxin-induced inflammation of mouse lung

Cycloheximide, which suppresses rat paw inflammation (Schayer & Reilly, 1968) was tested for effect on mouse lung inflammation. Mice were treated intranasally with saline, endotoxin, or endotoxin plus cycloheximide, killed 24 hr later and lungs weighed. Lungs of comparable weight were pooled in batches of three and assayed for histidine decarboxylase activity. Experimental conditions and results are shown in Table 2.

Experiment 5 : test for systemic effect of intranasal endotoxin

Endotoxin, given systemically to mice, activates histidine decarboxylase in liver, lung, spleen, lymph nodes and other tissues which phagocytize endotoxin particles. The purpose of this experiment was to determine whether activation of lung histidine decarboxylase by intranasal endotoxin was related specifically to a local effect on lung, or simply part of a systemic effect. Mice were divided into three groups of twelve each ; mice of one group received endotoxin intranasally, 100 μg in 0·05 ml. saline ; mice of another group were injected intraperitoneally with endotoxin 100 μg in 0·20 ml. saline ; controls received 0·20 ml. saline intraperitoneally. Mice were killed 6 hr after treatment, and three pooled livers assayed. Liver was assayed because its histidine decarboxylase is readily activated by systemic endotoxin (Schayer, 1967). Enzyme activities, relative to controls arbitrarily set at 100 mean, were for controls 60, 100, 107 and 133 ; for mice receiving endotoxin intranasally values were 100, 158, 220 and 426 ; for mice receiving endotoxin intra-

TABLE 2. *Comparison of weight and histidine decarboxylase activity of lungs from mice treated intranasally with endotoxin, or endotoxin plus cycloheximide*

Control		Endotoxin		Endotoxin+cycloheximide	
Wt.*	HD†	Wt.	HD	Wt.	HD
0·72	477	0·90	6,140	0·86	5,860
0·75	542	0·96	6,620	0·87	5,300
0·78	700	0·99	13,900	0·89	3,560
0·82	344	1·03	20,500	0·93	4,410
0·87	636	1·10	22,500	0·95	6,450
				1·02	3,380

* Mean weight of three pooled lungs (expressed as % body weight); lungs of comparable weight selected for pooling.
† Corresponding histidine decarboxylase activity units.
Experimental: Saline, endotoxin 100 μg, or endotoxin 100 μg plus cycloheximide 700 μg instilled intranasally in mice which were killed 24 hr later.

peritoneally values were 1640, 1710, 1720 and 3090. The data suggest that only small amounts of intranasal endotoxin enter the circulation.

Experiment 6 : evidence for increased in vivo *histamine formation in lung of mice given endotoxin intranasally*

This experiment was designed to test whether increased lung histidine decarboxylase activity induced by intranasal endotoxin corresponds to increased histamine formation *in vivo*. Mice were given saline or endotoxin intranasally. After 20 hr all were injected intravenously with (^{14}C)-L-histidine, killed 4 min later and lungs assayed for (^{14}C)-L-histidine and (^{14}C)-histamine. Results are shown in Table 3.

Experiment 7 : effect of intranasal endotoxin on (^{14}C)-histamine content of lung of mice injected with (^{14}C)-histamine

The purpose of this experiment was to find if lungs exposed to endotoxin might have an increased ability to remove histamine from the blood. Experimental conditions and results are shown in Table 4.

TABLE 3. *Effect of intranasal endotoxin on levels of newly formed histamine in lung of mice*

	I	II	I/II
	(^{14}C)-Histamine (d.p.m. in entire tissue)	(^{14}C)-L-Histidine (free) (d.p.m. in entire tissue)	D.p.m. (^{14}C)-histamine per 10^8 d.p.m. (^{14}C)-L-histidine*
Control	27	160,000	0·17
	82	172,000	0·48
	92	179,000	0·51
	89	183,000	0·49
Endotoxin	692	204,000	3·4
	882	210,000	4·2
	1,150	237,000	4·9
	1,430	248,000	5·8

*I/II values are unchanged if data are expressed as d.p.m./g.
Experimental: Saline or endotoxin, 100 μg, instilled intranasally in mice. Twenty hours later each mouse injected intravenously with (^{14}C)-L-histidine, killed 4 min later, and pooled lungs of three mice assayed.

TABLE 4. *Effect of intranasal endotoxin, and endotoxin plus actinomycin on ^{14}C-histamine and total ^{14}C in lungs of mice injected with ^{14}C-histamine*

	(^{14}C)-Histamine (d.p.m. in entire tissue)	Total (^{14}C) (d.p.m. in entire tissue)	(^{14}C)-Histamine (% of total ^{14}C)
Control	1,500	36,300	5·7
	1,730	40,200	5·9
	1,500	39,600	5·2
	1,440	37,100	5·3
	1,990	48,200	5·6
Endotoxin	1,840	37,200	6·7
	1,840	40,500	6·2
	2,000	37,600	7·2
	1,700	42,400	5·5
	1,690	40,500	5·7

Experimental: Saline or endotoxin, 100 μg, instilled intranasally in mice. Twenty hours later each mouse injected intravenously with (^{14}C)-histamine, killed 4 min later, and pooled lungs of three mice assayed.

Discussion

Experiments 1–3 (Table 1)

Saline groups

Most histidine decarboxylase values are in the same range ; the only high values correspond to lungs of the heaviest weight and may relate to a natural lung inflammation or infection (Schayer, 1962).

Actinomycin groups

At 6 hr, there is no evident difference from controls ; at 24 and 48 hr small increases in histidine decarboxylase activity are observed.

Endotoxin groups

At 6 hr, lung weight is close to control levels. At 24 hr it is increased and at 48 hr increased even more ; similar results have been reported by others (Herrmann *et al.*, 1959).

Histidine decarboxylase is strongly activated by endotoxin at 6 hr ; activity is high at 24 hr but has dropped by 48 hr. The data imply that histidine decarboxylase activation precedes the main increase in lung weight, an observation consistent with an early role in endotoxin-induced mouse lung inflammation. At 48 hr, the time of maximal inflammation, other mechanisms must be sustaining the inflammatory reaction.

Endotoxin-actinomycin groups

At 6 hr lung weights are only slightly above control levels ; at 24 and 48 hr weight is strongly increased relative to controls and to endotoxin groups. In all experiments, some mice dosed with endotoxin-actinomycin mixtures died during the second night ; their lungs were liver coloured and often more than double the normal weight.

At 6 hr, histidine decarboxylase activities in the endotoxin-actinomycin group are roughly the same as in the endotoxin group. At 24 and 48 hr, in the endotoxin-actinomycin groups of Table 1, histidine decarboxylase activities are high in the heavier lungs ; in fact there is a rough correlation between weight and histidine decarboxylase activity, a relationship not observed in other groups.

At 48 hr the tendency of actinomycin to sustain high levels of histidine decarboxylase activity in endotoxin-treated mice is obvious ; this effect can be seen to a smaller extent in the 24 hr groups, particularly in experiment 1. Whatever may be the role of histidine decarboxylase in inflammation, its continued activation by actinomycin is certainly associated with a marked exacerbation of inflammation.

Actinomycin D may enhance histidine decarboxylase activation by inhibiting synthesis of RNA required for formation of a protein (enzyme ?) involved in deactivation of histidine decarboxylase. Protein synthesis associated with histidine decarboxylase activation appears to be directed by long-lived RNA, and hence not susceptible to strong inhibition by actinomycin (Schayer and Reilly, 1968).

The rough correlation between histidine decarboxylase activity and weight in the 24 and 48 hr endotoxin-actinomycin groups (Table 1) may arise as follows: in endotoxin-treated mice, histidine decarboxylase activity reaches a maximum at some undetermined time which may vary from mouse to mouse ; then activity starts to drop. At 24 hr activity may be increasing in some mice, near maximal in some, and perhaps falling in others. Addition of actinomycin to the endotoxin may retard histidine decarboxylase deactivation without materially affecting activation ; this action would tend to stabilize enzyme activity at or near its highest level.

It is known to clinicians who have used actinomycin D to treat patients with tumours that this substance is highly pro-inflammatory (Dr. Frederick Phillips, Sloan-Kettering Institute, personal communication, 1968). The present findings may, in part, explain these observations.

Experiment 4

The data of Table 2 suggest that cycloheximide reduces endotoxin-induction of histidine decarboxylase activity, and lung weight. Because of variability in the data, however, no conclusion on the histamine-weight relationship has been drawn. The main value of this experiment is to show that cycloheximide, which like actinomycin D is toxic and blocks induction of many enzymes, does not affect our test system as does actinomycin. Thus the experiment serves to emphasize the unusual aspect of actinomycin action.

Experiment 6

The data of Table 3 show that in mice injected 4 min before sacrifice with (^{14}C)-L-histidine, those pretreated with endotoxin intranasally contained more than 10 times as much (^{14}C)-histamine in lung than did controls. This increased (^{14}C)-histamine is not due to a comparable increase in lung concentration of the substrate, free (^{14}C)-L-histidine.

Experiment 7

The data of Table 4 show that there was no major difference between endotoxin-pretreated and control mice in either total ^{14}C, or (^{14}C)-histamine, in lung 4 min after injection of (^{14}C)-histamine. These data show no evidence that lung of endotoxin-treated mice handles blood borne histamine in an abnormal manner. Accordingly, the data of Table 3 are best interpreted as revealing an increased rate of histamine formation in lung of endotoxin-pretreated mice.

We previously showed that protein synthesis inhibitors, the only drugs known to block irritant-induced activation of histidine decarboxylase, also suppressed inflammation (Schayer & Reilly, 1968). We now have shown that actinomycin D, the only drug known which enhances irritant-induced activation of histidine decarboxylase, also enhances inflammation in mouse lung. Histidine decarboxylase activation is associated with inflammation ; it must be either a causative factor, or a related but non-participating event. We have provided evidence in this paper and elsewhere (Reilly & Schayer, 1968b) that histidine decarboxylase activation corresponds to increased histamine formation *in vivo*. Since the newly formed histamine of mouse lung is not firmly bound (Reilly & Schayer, 1968a) and since histamine can

cause microvascular dilatation characteristic of early inflammation (Schayer, 1968b), it is difficult to rationalize a non-participatory role.

Finally, we suggest that enhancement of endotoxin-induced lung inflammation by actinomycin D may provide a rigorous test system for other proposed mediators. If intranasal actinomycin D blocks formation of an alleged mediator, or fails to enhance its formation, the substance is of questionable importance to this type of inflammation.

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REFERENCES

GINSBERG, H. S. (1955). Suppression of influenza viral pneumonia in mice by the non-specific action of xeroxin. *J. Immunol.*, **75**, 430–440.

GROUPÉ, V., DOUGHERTY, R. M. & MANAKER, R. A. (1956). Comparative effects of hydrocortisone, a derived pyrido-pyrimidine, and xeroxin on pneumonia produced in mice by viral and bacterial toxin. *J. Bact.*, **72**, 604–609.

HERRMANN, E. C., JR., ENGLE, C. & PERLMAN, P. L. (1959). Action of anti-inflammatory agents on endotoxin-induced lung inflammation in mice. *Am. J. Physiol.*, **197**, 803–807.

REILLY, M. A. & SCHAYER, R. W. (1968a). Studies on the histidine–histamine relationship *in vivo*. *Br. J. Pharmac. Chemother.*, **32**, 567–574.

REILLY, M. A. & SCHAYER, R. W. (1968b). Further studies on the histidine–histamine relationship *in vivo*. *Br. J. Pharmac.*, **34**, 551–563.

SCHAYER, R. W. (1962). Evidence that induced histamine is an intrinsic regulator of the microcirculatory system. *Am. J. Physiol.*, **202**, 66–72.

SCHAYER, R. W. (1967). Evidence for inactivation of colloidal irritants by mouse liver *in vivo*. *J. Reticuloendothel. Soc.*, **4**, 1–9.

SCHAYER, R. W. (1968a). Determination of histidine decarboxylase activity. *Methods of Biochemical Analysis*, ed. Glick, D., vol. 16, pp. 273–291. New York: Interscience.

SCHAYER, R. W. (1968b). Histamine and a possible unity of autonomous microcirculatory dilator responses. *Med. Coll. Virginia Q.*, **4**, 101–106.

SCHAYER, R. W. & REILLY, M. A. (1968). Suppression of inflammation and histidine decarboxylase by protein synthesis inhibitors. *Am. J. Physiol.*, **215**, 472–476.

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