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Inhalation Toxicity Studies

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Pollutants that contaminate the environment because of industrial establishments, agricultural practices and health programmes can give rise to serious community air pollution problems leading to respiratory and other health hazards. From time to time several environmental pollution accidents have occurred such as the recent Bhopal tragedy of 1984 which pose several questions regarding exposure of man and animals to various chemical substances during occupational and other calamities. One question which comes to mind is the effect of exposure to excessive concentration of chemical pollutants for short and or long periods, their clinical symptoms and appropriate treatment in cases of fatalities. Another question is regarding the safety limit of daily intake of chemicals due to occupational exposure and the precautionary measures to avert industrial accidents. Establishment of respiratory hazards of airborne drugs, chemicals, pesticides, war gases, smokes etc. requires the specialised approach of inhalation toxicity studies. Though inhalation toxicity studies are difficult to conduct and interpret, they are essential for detecting toxicity to the respiratory tract including the nose, nasal sinuses, nasopharynx, larynx, trachea, bronchi and lungs. The study detects the toxicity of agents especially those whose principle

route of exposure is via inhalation. It also helps in establishing safety limits and the possible role of various types of chemicals in producing lung cancer. The inhalation toxicity studies are so devised as to simulate environmental conditions and establish industrial and general population exposure or tolerance limits of different pollutants. Levels for safe industrial practice are to be established to find whether chemicals can be used safely and the possible limits and conditions of safe use. Two important guidelines for maintenance of satisfactory working conditions and levels are, Threshold Limit Value (TLV) and Maximum Allowable Concentrate (MAC). TLV is time weighted average concentration of a chemical to which population/workers may be repeatedly exposed daily without any adverse effects. MAC is the threshold limit which should not be exceeded any time during a normal working day (working hours). TLV or MAC parameters can be established by inhalation toxicity studies for maintaining industrial and environmental safe levels. The study also investigates the possible mechanism of toxicity of inhaled materials and compare the effects with agents exposed through other routes. Air borne substances that enter lungs affect the local cells directly or are absorbed into the circulation thereby carrying the insult

directly to other organs without passing through the stage of detoxification in the liver unlike in the case of oral route of exposure where a chemical can be absorbed into blood and transported to the liver from where it is transported to other organs after being metabolised.

Factors that effect the deposition and retention of pollutants in the respiratory tract

The deposition, retention and clearance of inhaled particles depend on a number of factors (1, 2).

1. Physico-chemical parameters of the inhaled particles, such as size and shape, density, viscosity, the electrical charge on it, volatility, hygroscopicity, inertial forces, settlement by gravity, Brownian movement, solubility of the particle, degree of equilibrium attained in the body compartments, diffusion rates of vapours, duration of exposure, influence the deposition and retention of inhaled particles.

2. Anatomical differences amongst and between different species of animals and man.

3. Physiological factors such as breathing (nasal or by mouth), the rate of air flow, expansion and contraction during respiration, diameters of the airways, ventilation rates in athletes, age, body weight, all have influence on the deposition and retention of particles in the different parts of the respiratory passage.

4. Health status — Different pathological conditions prevailing in an indi-

vidual such as nasal blockage, excessive production of mucous, emphysema, pulmonary fibrosis, impairment of blood flow through lungs associated with respiratory tract ailments influence the deposition and retention of particles.

5. Dose — In the usual toxicological jargon, dose is often expressed in terms of mg/kg i.e. mass of material introduced into the animal species divided by the body weight. However in inhalation toxicity the amount of inhaled material entering the internal milieu of the body depends on the concentration and particle size of the inhaled material as well as the duration of exposure, the type of breathing exhibited by the test animals. Thus the dose is based on exposure condition and the lethality is expressed frequently as Median Lethal Concentration (LC_{50}), that is the concentration which kills 50% of a group of test animals within a fixed period of time after exposure. Concentration in the chamber is expressed as milligram of compound per cubic meter of air (mg/m^3) or gases can be expressed in terms of parts by volume per million parts of air (p.p.m.).

Choice of species

One of the most important aspects in any toxicological study is the selection of appropriate model that would give the closest interpretation of the data applicable to human being (3). The ideal situation would be man as a model. But because of ethical considerations and where toxicological hazards of unknown pollutants which have not been well defined, the exposure of human volunteer is not practical. It is for this reason that most of the interpretations and possible risks to human are still based on animal.

studies. Rodents such as rats, mice, hamsters, guineapigs, rabbits and non rodents like dogs, cats, swine have all been used in the evaluation of inhalation toxicity. Primates have also been used as a model. However considering the comparative morphology of the respiratory tract and similarity to responses with man the horse is the ideal model. The mechanisms and organisation of structure in the lungs of horses are similar to man and hence horse is a suitable model for studying the specific respiratory toxicity (2, 3, 4, 5).

Exposure chambers for housing the animals

The design of the exposure chambers into which experimental animals are introduced will depend on the inhalation system one desires. Housing the inhalation chambers itself requires buildings, preferably designed for installation of the inhalation toxicity units. The rooms for housing exposure chambers should be with high ceiling with adequate floor space so as to accommodate the chambers and related equipments like aerosol and vapour generators, filters, flow filters. There should be cleaned filtered air, with temperature and humidity controls in the chambers and in the room housing the chambers.

Static exposure system — This system refers to exposure of animals to static atmosphere where known amount of agent is introduced into an enclosed chamber and mixed. There are limitations in this type of set up because of progressive deterioration in the atmosphere of the enclosed chamber due to gradual depletion of oxygen, accumulation of carbon dioxide and moisture, and increase

in the temperature inside the chamber. In spite of these limitations the static system is still of practical importance for assaying acute toxicity of limited material specially biological aerosols.

Dynamic exposure system — In the dynamic system there is a continuous introduction of air flow and the test material. The animals are exposed in chambers through which passes air, containing the test material. The air flow through the chamber should be such as to maintain the concentration of water vapour and carbondioxide, and temperature within suitable limits. The flow of air should be sufficient to ensure that animals under experiment are exposed to constant concentration of the test material. To ensure a constant concentration, frequent monitoring by an appropriate instrument or technique is required so as to regulate the concentration, because there is a tendency for the concentration of the test substance to be lowered in normal process due to adsorption on the walls of the chamber, losses on the skin and fur of the animals, and uptake by test animals. The principles involved in maintaining concentration in the chamber and the flow rate has been reviewed (6).

Whole body exposure chamber — The chamber must be big enough to expose sufficient number of animals. The size of the chamber depends on the animal model, the number of animals to be used and the parts of the body to be exposed. The total animal volume usually does not exceed 5% of the total chamber volume. The chambers are usually made of stainless steel and glass, though other types of material such as aluminium,

wood, wood coated with epoxy paint have been used. Inhalation chambers are equipped with instruments for monitoring and control of temperature, humidity, air flow and mechanism to ensure uniform distribution of aerosol. The advantages of selecting chambers made of stainless steel are that it does not react with the test material and are easy to clean. The two sides of the chamber are made of glass or transparent material in order to view the experimental animals during exposure. The air flow in the chambers must be accurately controlled. Generally filtered conditioned air is supplied in excess of that required and then tap of the common supply for each chamber. The ratio of chamber volume to air flow mostly ranges from 1 to about 6. The chambers must be safe for laboratory staff while handling toxic substances. Chambers are operated at negative pressure to ensure that any leaks draw air into the system. The chambers must be cleaned thoroughly and the effluents decontaminated before being released into the environment. The animals while under exposure in the chamber must be provided with food and water. Provision for cleaning the chamber specially when animals are under exposure for long duration should also be there. The animal cages in the chamber should have all six sides made of stainless steel wire mesh to ensure proper mixing. The advantages of whole body exposure chambers are that large number of animals can be simultaneously exposed with minimal physiological stress. The movements of the animals are more free. The disadvantages being that large quantities of test material is required. There is also deposition of air borne material on the fur of the animals with

chances of possible absorption through the skin and ingestion by licking.

Head and nose exposure system — The exposure of only head and nose for inhalation toxicity studies has its own advantages. Nose exposure system have been designed and widely used specially for effects due to exposure to radioactive aerosols and cigarette smoke. The difficulty in exposing animals to cigarette smoke has resulted in the development of a number of nasal exposure system by various laboratories (2).

The major advantage of head nose exposure units is that the exposure of agents to dermal and oral routes is reduced as there is no contamination to the body fur of the animals. Also, one requires less quantities of test substances. Nose exposure system is ideal to a situation where skin absorption is not desirable, the material is hazardous and to study acute effects. However the disadvantage of stress to animals is more than in whole body exposure chambers as they are kept in holders which may also affect the respiration pattern.

It is essential to stress that during operation of either type of chamber system a number of variables have to be monitored. Firstly, the concentration of the pollutant in the chamber be checked. Secondly, the particle size should be monitored. Thirdly, the flow rate of air and the pollutant, and the pressure in the chamber should be checked and recorded frequently. Fourthly, it is also important to monitor the temperature and humidity of the chamber.

Human exposure studies — The ultimate goal of all toxicity studies is to

interpret the possible hazards to human subjects. The best way to do it, is to use human models. However ethical problems will always limit human exposure studies to primarily evaluation of unknown pollutant first in animals and then wherever possible to study carefully controlled exposure of human beings to toxicants at low or safe concentrations. In this context, information on dose response can be obtained through epidemiological studies in exposed population. Modern facilities for human exposure studies are in the form of completely air conditioned rooms operated at slightly negative pressure. The volunteers are required to sit comfortably in chairs and are provided with tables. The volunteers are provided meals and beverages during exposure. The subjects under exposure are continuously under expert medical vigilance and their activities are monitored. Such study facilities are linked to exposure to low concentration or concentrations considered to be safe in order to study the psychomotor effects or for establishing maximum permissible concentration.

Generation and control of contaminant concentration

There are various methods for generating vapours and particles (2). Flow-dilution devices are used for generating vapours. In case of test material which is a liquid at room temperature, the vapourization of the liquid is required. Of the several procedures adopted to vaporize, one is by using motor driven syringe to apply the liquid to a heated plate in a calibrated stream of air. In another method the airstream is saturated with vapour and then diluted with air to obtain the desired concentration. Yet another technique uses permeation tubes.

Particles can be generated from a dry powder or from a liquid. The size of the particle generated can either be heterogeneous (unequal size) or monodisperse (uniform size). An instrument for generating aerosols from dry powders is the Wright dust feed. For liquid test material, droplets are formed with the help of wet dispersion generators. There are air-driven generators known as nebulizers that produce droplets of different sizes and disperse. Dispersal of droplets of uniform size is often required when studying required deposition. Uniform droplets are achieved with the help of spinning disc generators or by device employing controlled condensation process.

The choice of methodology used for concentration monitoring of contaminants in the chamber air sample, depends on the sampling technique, equipment used for analysis and the time of analysis. The method for collection of gaseous sample involves the use of a gas collector like an evacuated flask or bottle for collecting a definite volume of air at known temperature and pressure, or allowing a known volume of sample air to pass through a collecting medium to traps and removal of the desired contaminants from the sample air. The actual detection and analysis is done by principles using colorimeter, spectrophotometer and infrared spectrophotometry etc.

In case of particulate material one determines its mass concentration and size. Concentration can be determined by, first collecting the particle material and then analysing by chemical methods as in gaseous sample. The technique

used for collecting, includes filtration, thermal and electric precipitation and sedimentation. For measuring particle size direct method like conventional or electron microscopy can be used or measured indirectly by using cascade impaction and light scattering devices.

The inhalation toxicity tests and maintenance of the animals in the exposure chambers

The duration and maintenance of experimental animals in the exposure chambers depends on whether the toxicity study is of a single or few repeated exposures. Three types of studies are carried out, namely, acute, subchronic and chronic. In acute inhalation toxicity study, the animals are subjected to a single exposure or repeated exposures of not more than 8 hours. When repeated exposure is done the animals can be removed from the chambers to other accommodation for the over-night period. Acute toxicity studies provide information on the immediate adverse effects, the minimum lethal concentration, the LC_{50} and information for designing subchronic or chronic studies.

The duration for chronic inhalation studies, vary from 3-4 months continuous exposure to even upto 5 years. The animals are given daily exposure of 6-7 hours, 5 days a week (intermittent exposure) to study the occupational effects or 22-24 hours of exposure per day, 7 days a week (continuous exposure) to assess the impact of environmental exposure. The main objective of subchronic or chronic studies is to ascertain the adverse effect of repeated insults as is encountered in occupational exposure to hazardous pollutants. The

study will bring out information on the effects on various body systems, the effects on various individual target organs and will help in determining the safe levels of exposure. It will also help in understanding the mechanism of action. Chronic inhalation toxicity studies may correspond to life long exposure (life span) and are significant in assessing carcinogenic potential of the toxicant.

Assessment of toxicity changes

In order to assess the toxicity, the observation and examination of experimental animals during and after the exposure for toxic effects follows the routine procedures as in studies by other routes except that more attention is paid to the effects on the lungs. The usual parameters such as changes in body weights, mortality, organ to body weight ratio, behaviour, food and water consumption, various haematological and biochemical indices and postmortem findings (gross and histopathological) are studied. The biological changes that are particularly useful in inhalation toxicity studies are the morphological, functional and biochemical changes in the lungs. The morphological changes in the lungs will depend on the concentration of the inhaled material and the duration of exposure. Short term exposure to high concentration may result in acute changes in the lungs like oedema, necrosis and purulent inflammation. The inhalation at lower concentration for longer duration may result in chronic changes in the lungs such as fibrosis and even neoplasia. The functional changes include the alterations in respiratory functions such as frequency, rhythm, alteration in vital capacity, gaseous

transport across the pulmonary epithelium etc. The biochemical changes in the lungs are of importance for evaluation of pulmonary toxicity, the capacity of lungs for biotransformation of foreign chemicals and the role of cytochrome p-450 dependent enzyme system in pulmonary microsomes(2).

Conclusion

One of the major routes through which toxic material can enter our body is by inhalation. The present knowledge regarding the physico-chemical properties of aerosols and particle clouds their generation, the comparative anatomy and physiology of the various animal models have made controlled inhalation toxicity studies possible and more meaningful in terms of extrapolation of these toxicity studies to possible human situation. As of today few institutions around the world have the necessary equipment and facilities for conducting inhalation toxicity studies. There is a serious shortage of facilities in our country for both short term and long term inhalation toxicity

experiments. Some of the basic requirements for carrying out inhalation toxicity such as inhalation chambers, generation of aerosols and particle clouds, instruments for monitoring exposures, appropriate disease free animal models, methods for measuring lung function, adequate facilities for histopathological examination of exposed specimens are needed for successfully carrying out inhalation toxicity studies. With the advancement of technology to meet the ever increasing demand for fulfilling the basic needs and comforts of human being, environmental pollution in some form or the other seems to be a part of life. Therefore it is important to set up inhalation toxicity facilities in more centres and train scientists with basic toxicology background for conducting inhalation toxicity studies in order to assess the impact of environmental pollution on health of humans, animals and other living systems and formulate safety measures so as to prevent future environmental toxicity problems.

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Synthesis and Study of 6,7-Dimethoxy-3-Substituted-4-Imino-2-Thiotetrahydroquinazolines as Antituberculous Agents

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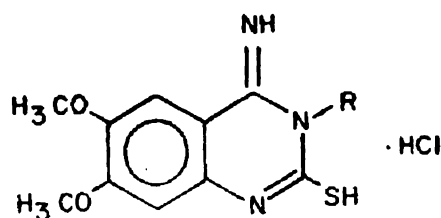
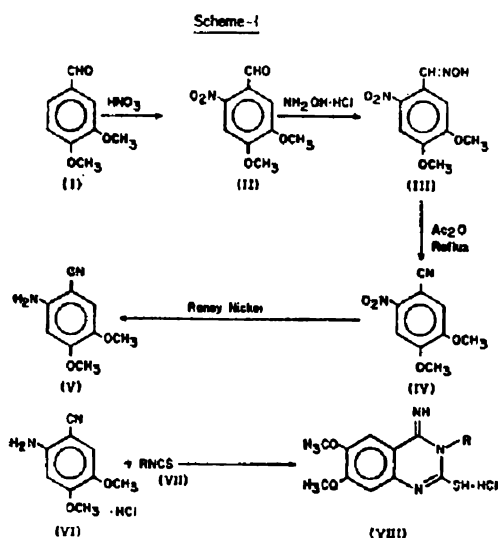
ABSTRACT

3,4-Dimethoxy-6-aminobenzonitrile hydrochloride (VI) reacts with different alkyl or substituted (VII) aryl isothiocyanates in reflux temperature to yield 6,7-dimethoxy-3-substituted-4-amino-2-thiotetrahydroquinazoline (VIII). These compounds were tested against *M. tuberculosis* H₃₇Rv strain, using serial dilution technique. The presence of alkyl or aryl substituent in the quinazolium part of the molecule does not have much favourable effect for biological activity.

Various quinazoline compounds are reported to possess antibacterial and antimalarial properties. For example, 2-(chlorophenyl)-6,7-dimethoxy-4-hydroxy quinazoline has been reported to have antimalarial activity (1). Our efforts were directed to synthesize similar compounds in the 6,7-dimethoxy quinazoline series and test them for antibacterial, antitubercular and allied activities.

Chemistry

The presence of methoxy groups in a fused quinoline system is known to impart antimalarial activity to the ring system (2). Efforts are made in this communication to synthesize tricyclic quinazoline ring system with two methoxy groups at 6 and 7 position of the heterocyclic nucleus. The required starting material i.e., 3,4-dimethoxy-6-aminobenzonitrile was prepared from veratraldehyde, as shown in Scheme 1. However, reduction of the 5-nitro group of compound No. IV was carried out with Raney Nickel in a Parr hydrogenation apparatus as described in (1), and gave almost quantitative yields with clean products.



Where, R = Alkyl or substituted-aryl.

Fig.-1

The alkyl and substituted aryl isothiocyanates required for synthetic work were prepared by known methods (3). 3,4-Dimethoxy-6-amino benzonitrile was converted to the hydrochloride and then reacted with different alkyl or substituted aryl isothiocyanates in refluxing ethanol as reported by Paur et al. (4).

Experimental Procedure

6,7-Dimethoxy-3-allyl-4-imino-2-thiotetrahydroquinazoline hydrochloride — 3,4-Dimethoxy-6-aminobenzonitrile hydrochloride (0.815 g; 0.0037 mole) was added to freshly prepared allyl isothiocyanate (0.366 g; 0.0037 mole). Into this mixture ethanol (20 ml) was added

and the reaction mixture was refluxed for 6-8 hours. The crude product was precipitated out at the end of the reaction. The product was filtered and washed with ethanol (after cooling). The compound was recrystallized from DMF. Yield: 0.77 g (65.0%); m.p. 48.5°C.

The purity of the compound was confirmed by TLC (MeOH-CHCl₃-C₆H₅: 70:20:10).

Analysis for C₁₃H₁₅N₃O₂S. HCl:

Calcd. C, 49.74; H, 4.78; N, 13.39%

Found C, 49.70; H, 4.83; N, 13.00%

All the quinazoline derivatives were prepared by a similar procedure and their relevant data reported in Table 1.

Table 1
6, 7-Dimethoxy-3-substituted-4-imino-2-thiotetrahydroquinazoline

No.	R	Mol. formula	M. p. * °C	Yield %	Antituber- culosis activity** (mcg/ml)
1.	C ₂ H ₅	C ₁₂ H ₁₆ ClN ₃ O ₂ S		52	—
2.	n-C ₃ H ₇	C ₁₃ H ₁₈ ClN ₃ O ₂ S		93	—
3.	iso-C ₃ H ₇	C ₁₃ H ₁₈ ClN ₃ O ₂ S		37	—
4.	n-C ₄ H ₉	C ₁₄ H ₂₀ ClN ₃ O ₂ S		48	12.5
5.	iso-C ₄ H ₉	C ₁₄ H ₂₀ ClN ₃ O ₂ S		58	12.5
6.	Cyclo-C ₆ H ₁₁	C ₁₆ H ₂₂ ClN ₃ O ₂ S		32	12.5
7.	Alkyl	C ₁₃ H ₁₆ ClN ₃ O ₂ S	48.5	65	12.5
8.	n-C ₆ H ₁₃	C ₁₆ H ₂₄ ClN ₃ O ₂ S		58	—
9.	C ₆ H ₅	C ₁₅ H ₁₆ ClN ₃ O ₂ S	173	62	—
10.	C ₆ H ₅ CH ₂	C ₁₇ H ₁₈ ClN ₃ O ₂ S	195	68	—
11.	p-BrC ₆ H ₄	C ₁₆ H ₁₅ BrClN ₃ O ₂ S	252	64	—
12.	p-ClC ₆ H ₄	C ₁₆ H ₁₅ Cl ₂ N ₃ O ₂ S	266	57	12.5
13.	p-CH ₃ C ₆ H ₄	C ₁₇ H ₁₈ ClN ₃ O ₂ S	152	60	—
14.	p-CH ₃ OC ₆ H ₄	C ₁₇ H ₁₈ ClN ₃ O ₂ S	175	54	—
15.	o-CH ₃ OC ₆ H ₄	C ₁₇ H ₁₈ ClN ₃ O ₃ S	92	55	12.5
16.	p-C ₂ H ₅ OC ₆ H ₄	C ₁₈ H ₂₀ ClN ₃ O ₃ S	205	67	—
17.	p-(CH ₃) ₂ C ₆ H ₄	C ₁₈ H ₂₁ ClN ₃ O ₂ S	275	63	—

* Melting points were taken in open capillary tubes and are uncorrected.

** Minimum inhibitory concentration in vitro anti-T.B. action for Isoniazid = 0.04 mcg/ml and for ethambutol = 0.15 mcg/ml. — = Inactive at 12.5 mcg/ml.

R = See Fig. 1.

Results and discussion

In vitro antitubercular testing was carried out against virulent strain of *M. tuberculosis* (H₃₇Rv) by the method of disperse culture technique (5) using Kirchner's Medium (6).

Seventeen compounds were prepared and tested against bacteria, fungi and *M. tuberculosis* H₃₇Rv. Compounds Nos. 4, 5, 6, 7, 12 and 15 (Table 1) showed antitubercular activity at 12.5 mcg/ml; however, at lower dilution no activity was observed. Other compounds did not show any activity even upto 100 mcg/ml dilution.

The compounds under test were also screened *in vitro* against *Salmonella typhi* (RW 786 strain) and *Staphylococcus aureus* (strain 209). The screening was

done by serial dilution method on Nutrient broth medium (7).

The antifungal screening was done against *Trichophyton rubrum* and *Trichophyton mentagrophytes*. The liquid medium described by Schraustatter *et al.* (8) was used for present screening (7).

The presence of alkyl or substituted aryl molecules does not make any difference in the biological properties of the compounds. When tested for antibacterial and antifungal properties none of the compounds exhibited any biological properties.

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Evaluation of Mosquito Larvicidal Activity of Petroleum Ether Extracts of Indigenous Plants *Vitex Negundo*, *Bryonopsis Laciniosa* and *Porana Racemosa*

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ABSTRACT:

Vitex negundo Linn leaves, *Bryonopsis laciniosa* Linn and *Porana racemosa* Roxb. were collected from Poona and Satara Districts, dried in shade, powdered to uniform mesh and extracted with petroleum ether in Soxhlet. These extracts free of solvent were evaluated for mosquito larvicidal activity (*Culex P. fatigans*). Promising results were observed.

Introduction

Vitex negundo Linn (Nirgundi), is found throughout in India in the warmer zone. The leaves are reported to have many medicinal properties. Juice of the leaves is used for removing worms from ulcers and the oil is applied to sinuses and sores. Fresh leaves are reported to give 0.05% of essential oil. An alkaloid Nishindine is isolated from leaves (1). The leaves are also reported to have insecticidal activity. Madan et al have reported pharmacognostic study of leaf and root (2). Many authors have reported phytochemical investigation of various parts of *Vitex negundo* Linn (3-5).

Bryonopsis laciniosa Linn (Shivlingi), is found throughout India. Leaves are applied to soothe inflammation. The plant is bitter and its decoction is used in the traditional system of medicine as tonic. It contains bryonin, a bitter principle (6).

Porana racemosa Roxb. (Bhovari), is found throughout India, mostly in the western ghats.

Material and Methods

1.0 kg of shade dried plant material was powdered to uniform mesh and then extracted in Soxhlet with petroleum ether (40-60°). The extracts were dried on water-bath and weighed. It was found that the leaves of the three above mentioned plants gave 1.66%, 1.79% and 1.34% (w/w) of extract respectively. The extracts were evaluated for activity as recommended by the WHO expert committee on insecticides (7).

The fourth instar larvae of *Culex P. fatigans* were used throughout the experiments. All experimental exposures were done in 500 ml glass beakers. Twenty five larvae were placed in 250 ml tap water, containing various concentrations of the extracts and 24 hr mortality was

observed. The residual activity of the extracts was also studied after 24 and 48 hours.

Results and Discussion

From Table I, it can be seen that 0.1% of all extracts produced 100% mortality of larvae of *Culex P. fatigans*, whereas leaves of *V. negundo* in 0.01% concentration, of *P. racemosa* in 0.025% concentration and of *B. laciniosa* in 0.02% concentration produced 36%, 16% and 28% mortality respectively.

Table II shows residual activity of petroleum ether extract of *Vitex negundo* leaves. It can be seen that the activity

had considerably reduced at the end of 48 hrs, but data in Tables III and IV show that residual activity of *Bryonopsis laciniosa* Linn and *Porana racemosa* Roxb. was quite promising at 0.5% concentration. Activity was seen upto 96 hrs. It is felt that these results are extremely encouraging.

Acknowledgement

Authors are grateful to Dr. V. R. Deshpande, Director, Haffkine Institute for his keen interest in this work. Thanks are also due to Dr. D. M. Renapurkar, Assistant Director, Department of Zoonosis for his co-operation.

Table I

Culex P. fatigans larvicidal activity of petroleum ether extracts

No.	% Conc.	% Mortality (24 hrs)		
		<i>Vitex negundo</i>	<i>Bryonopsis laciniosa</i>	<i>Porana racemosa</i>
1.	0.01	35	ND	ND
2.	0.025	ND	28	16
3.	0.05	100	84	88
4.	0.1	100	100	100
	Control	Nil	Nil	Nil

ND: Not done

Table III

Residual larvicidal activity of petroleum ether extract of *Bryonopsis laciniosa*

% Conc.	% Mortality (hours)			
	24	48	72	96
0.125	100	100	80	28
0.250	100	100	80	80
0.5	100	100	100	84
1.0	100	100	100	96
Control	Nil	Nil	Nil	Nil

Table II

Residual larvicidal activity of petroleum ether extract of *Vitex negundo* Linn leaves

No.	% Conc.	% Mortality (hours)	
		24	48
1.	0.05	100	56
2.	0.1	100	68
	Control	Nil	Nil

Table IV

Residual larvicidal activity of petroleum ether extract of *Porana racemosa*

% Conc.	% Mortality (hours)			
	24	48	72	96
0.5	100	100	100	92
1.0	100	100	100	100
Control	Nil	Nil	Nil	Nil

(Continued on page 15)

Observations on Periodicity of output of schistosome eggs in the urine samples of schistosomiasis Cases

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Introduction

In the course of surveys for schistosomiasis, in the village Gimvi (1), it was observed that the morning specimens of urine showed only red blood cells. The presence of schistosome eggs was noticed only when the samples were collected early in the afternoon. A study in urinary schistosomiasis was therefore carried out to investigate whether there was any periodicity in the egg output. The results are presented in the present paper.

Material and Methods

Repeated urine examinations were undertaken in three male patients known to excrete schistosome eggs for a period of six days. Samples of urine (entire bladder contents) were collected in conical flask as per method described (2). These samples were collected at different hours i.e. 6-8 a.m., 8-10 a.m., 10-12 a.m., 12-2 p.m., 2-4 p.m. and 4-6 p.m. regularly for six study days. The urine samples were observed for schistosome ova as per methods already described (3, 4).

Results and Discussion

There was considerable variation in the egg output, the maximum excretion was observed during the period from 12 noon to 2 p.m. while the lowest excretion was observed between 6 t. 12 p.m. and 2 to 6 p.m.

The main obstacles in the surveys and clinical evaluation of the disease are the great variability in the egg output in *S. haematobium* infections and the difficulty in translating clinical symptoms into numerical terms, with the result that no co-relation can be made between worm load and survey of clinical disease. Scott (5), has suggested that the problem of variability in egg output could be overcome if specimens were collected at a particular time of day, and if emphasis were placed on a group of people rather than on individual persons. Our data supports this view.

Acknowledgement

We are grateful to the Director, Haffkine Institute, Bombay for providing excellent facilities during the field study.

Table 1

**Periodicity of output of schistosome eggs in the urine samples
of infected persons observed in 1982**

Sr.	Sex	Age	I day	II day	III day	IV day	V day	VI day
			6-8 a.m.	8-10 a.m.	10-12 p.m.	12-2 p.m.	2-4 p.m.	4-6 p.m.
1	M	10	0	2	4	6	3	0
2	M	12	0	4	10	15	5	0
3	M	32	0	0	1	2	1	0

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LARVICIDAL ACTIVITY

(Continued from page 13)

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Effect of Anti-diabetic drugs on serum, liver and Aortic Lipids in adult Male Albino Rabbits

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and

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Adult albino rabbits were treated with insulin and tolbutamide for 3 months, with and without cholesterol feeding, along with their controls, to study the effect of anti-diabetic drugs on lipid pattern. Analysis of serum samples showed higher cholesterol levels at the end of the experiment ($p < 0.005$), in case of tolbutamide treated group. However, in case of cholesterol fed groups, insulin group showed higher cholesterol levels as compared to controls ($p < 0.005$) as well as tolbutamide ($p < 0.005$); serum phospholipid levels were higher in all the groups as compared to their respective controls. Serum triglyceride levels did not show any increase after treatment in all the groups. Liver analysis showed higher total lipid content in case of insulin treated group (without cholesterol) than in control ($p < 0.025$). There was no significant difference in case of total cholesterol and phospholipid content of the liver among all the groups. Free cholesterol content of the liver was higher in case of insulin treated groups with and without cholesterol feeding. Aortic total lipids and total cholesterol contents were higher in case of insulin treated groups without cholesterol treatment than in control ($p < 0.05$). Present study indicates lipogenic behaviour of tolbutamide and insulin.

Introduction

Hyperlipidemia is a common finding in diabetes mellitus and cardiovascular diseases. Several studies have shown altered lipid profile in patients taking antidiabetic drugs for a long time (1, 2, 3). However, some workers have shown no change in the lipid levels after long term treatment with antidiabetic drugs (3, 4, 5). Experimental evidence suggests that sulfonylurea compounds induce lipogenesis and rise in serum cholesterol. There is an indirect evidence that insulin also has an effect on arterial lipid metabolism. Vascular lesions similar to the earlier lesions in human atherosclerosis have been shown in chicks after treatment with long acting insulin (6, 7). The present study depicts long term effect of insulin and tolbutamide on lipid metabolism in relation to atherogenesis with and without cholesterol feeding in adult albino rabbits.

Materials and Methods

Thirty six adult male albino rabbits Haffkine bread strain weighing between 1.5 to 2 kg were caged individually and divided into 2 sets (A and B). Set A consisted of 3 groups i.e. 1. Control group. 2. Insulin treated group. 3. Tolbutamide treated group. Set B consisted of three groups i.e. 1. Control group, receiving only cholesterol, 2. Insulin treated group

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receiving insulin and cholesterol. 3. Group receiving cholesterol and tolbutamide. Animals in set B received 0.7 gm of cholesterol/kg body weight/day in the form of thick suspension in groundnut oil by oral route. Insulin treated animals in both the sets received 0.03 unit/kg body weight/day. The dosage which produced maximum fall of 10 to 20 mg per 100 ml of blood sugar was selected for the experiment in order to maintain minimum degree of hypoglycemia. Tolbutamide dose was also selected in a similar manner. Tolbutamide groups received 50 mg/kg body weight/day. All animals were fed *ad libitum* the standard stock diet consisting of lucern grass, green bengal gram, cereal bran and carrots. Tolbutamide powder was supplied by the department of Chemotherapy, Haffkine Institute, Bombay and insulin was purchased from — Boots laboratories, Bombay. Cholesterol was obtained from J. T. Bakers, Germany.

Treatment was carried out for 3 months. At the end of twelve weeks, animals were sacrificed after 36 hours fasting and blood, liver and aorta were removed for lipid studies. Total lipids, cholesterol (free and total), triglyceride and phospholipids were estimated from liver, serum and aorta. Total lipids were quantitatively extracted with chloroform and methanol (2:1) according to the procedure of Folch *et al* (8) and the extracts were made to a known volume. Modified method of Abell *et. al.* (9) was followed for cholesterol estimation. Lipid phosphorus was estimated by the standard method of Fiske and Subbarao (10). Triglycerides were estimated from the serum by method of Carlson (11). Total lipids were extracted as described

above. The residue which contained total lipid was weighed and the amount of total lipid was calculated and results were statistically analysed using student's 't' test.

Results

As observed in Table-1 the tolbutamide group (Group III) showed significantly higher serum cholesterol levels at the end of the experiment ($p < 0.005$) as compared to the initial levels, whereas insulin treated group (Group II) showed significant decrease as compared to the initial level at the end of the experiments ($p < 0.025$). Serum phospholipids increased in all groups except control group. Final levels of serum cholesterol were significantly higher in Group III than in Group II ($p < 0.001$). In cholesterol fed groups (set B), insulin treated group (Group V) showed higher cholesterol levels than the control group ($p < 0.002$) as well as the tolbutamide group ($p < 0.005$). Serum phospholipids levels were significantly higher in insulin treated group (Group II) and tolbutamide group (Group III) than in control group ($p < 0.005$ and $p < 0.001$ respectively). Tolbutamide group also showed significantly higher levels of serum phospholipids than the control group ($p < 0.002$). In cholesterol fed animals (Set B) also, the insulin group and the tolbutamide group had significantly higher levels as compared to controls ($p < 0.001$ and $p < 0.05$ respectively).

Serum triglyceride levels did not differ significantly in any of the groups. As observed in Table 2, Group II had higher liver weights than Group I ($p < 0.05$). Group II also showed significantly higher total lipids as compared to Group I

($p < 0.25$). Free Cholesterol in liver was significantly higher in Group III as compared to Group I ($p < 0.025$). Similar trend was also observed in set 'B' where free cholesterol level of Group VI was higher than Group IV and Group V ($p < 0.001$ and $p < 0.0025$, respectively). No significant difference in the total cholesterol and phospholipid contents of the liver were found in set 'A' as well as in 'B'.

Aortal lipid levels were higher in case of insulin group as compared to control group ($p < 0.05$) in case of set 'A' whereas no significant difference was observed in any of the groups (Table 2) in set B. Total cholesterol levels of aorta in case of Group II showed higher statistically significant differences as compared to Group I in set 'A' ($p < 0.05$). No significant difference was found in any of the other groups.

Discussion

The present data on the lipid levels of the drug treated rabbits, did show some differences in the lipid levels of tolbutamide and insulin treated animals in both the sets of experiments. Serum cholesterol levels were significantly higher in Group III (Table 1) than in Group I and II ($p < 0.001$) in both the cases. Studies conducted earlier in this laboratory on well controlled diabetic patients, also showed hypercholesterolemia after long term tolbutamide therapy. Total lipid levels were significantly higher in the livers of insulin treated group than control group ($p < 0.025$). Heavier livers observed in this group could be perhaps due to higher total lipid in the liver in the same group which was probably because of lipogenic action of insulin. Lipogenic behaviour of insulin is further confirmed by the higher chole-

sterol and total lipid levels in case of insulin treated groups as compared to their controls in sets A and B. Significant increase in the cholesterol content of liver in experimental group was not found. This could be attributed to short duration and smaller doses of the drugs. The exact site of action of insulin on metabolism of cholesterol was not very clear till the last decade. Nepkroeff et. al. (12) however reported increase in hepatic B-hydroxy-B-methyl-glutaryl CoA reductase activity on insulin treatment in rats.

Increase in cholesterol and total lipid content of aorta in insulin group (Table 2) indicates the lipogenic action of insulin in aorta. Increased arterial cholesterol synthesis was found after insulin treatment by Mahler (13) and Stout (14). Earlier the dependence of aortic wall metabolism on insulin (14) was pointed out by some workers (15). These findings are of interest when one considers some adverse clinical findings in diabetic patients treated with these drugs over a long time. Higher incidence of atherosclerosis is found in people who have abnormally large amount of insulin and excessive insulin levels have been reported in vascular disease (16, 17, 18). Excessive insulin promotes lipogenesis and arterial wall participates in it. Present experiments report induction of lipid accumulation in the arteries of the experimental animals even without dietary manipulation. Strong need for the search for harmless, yet more helpful remedy in controlling diabetes mellitus is suggested.

Acknowledgements

The author is grateful to the Director, Haffkine Institute for providing excellent facilities to conduct this study.

Table 1

Mean blood sugar, serum cholesterol and phospholipid levels at various intervals in insulin/tolbutamide treated rabbits

		0 week			13th week		
		Sugar mg/100 ml	Cholesterol mg/100 ml	Phospho- lipids mg/100 ml	Sugar mg/100 ml	Cholesterol mg/100 ml	Phospho- lipids mg/100 ml
Set 'A'							
I. Control	n-6	65.3 ± 17.4	60.3 ± 19.9	65.0 ± 24.5	75.3 ± 9.4	41 ± 7.9	75 ± 21.5
II. Insulin	n-6	74.0 ± 15.0	50.6 ± 11.3	82.0 ± 26.7	60.0 ± 13.8	36 ± 3.0	133 ± 29.0
III. Tolbutamide	n-6	45.6 ± 10.9	43.0 ± 15.4	48.3 ± 15.0	52.0 ± 14.0	70 ± 6.2	212 ± 18.0
Set 'B'							
IV. Control + Cholesterol	n-6	68.0 ± 17.2	41.6 ± 3.2	73.3 ± 13.3	53.3 ± 4.8	1046 ± 126.0	145 ± 43.0
V. Insulin + Cholesterol	n-6	57.3 ± 13.5	50.7 ± 11.7	57.0 ± 24.8	62.8 ± 17.3	1265 ± 69.0	312 ± 39.9
VI. Tolbutamide + Cholesterol	n-6	57.8 ± 14.3	51.8 ± 5.4	123.0 ± 21.7	61.3 ± 14.9	1001 ± 133.0	210 ± 49.4

Values are means $\pm$ S.D.

Table 2

Mean liver weights, total fat, free and total cholesterol, phospholipids and triglycerides of insulin/tolbutamide treated rabbits.

Groups	Liver weights gm/100 ml	Total lipids gm %	Liver		Phospho- lipids mg %	Aorta Total lipids gm/100 gm of tissue	Total Cholesterol mg/100 gm of tissue
			Cholesterol				
			Free mg %	Total mg %			
Set 'A'							
I. Control	45.8 ± 7.2 (6)	4.42 ± 0.28 (6)	135 ± 37.6 (6)	254 ± 52.5 (6)	1239 ± 569 (6)	11.1 ± 4.2 (6)	54.0 ± 32.0 (6)
II. Insulin	52.7 ± 1.9 (6)	8.11 ± 1.26 (6)	161 ± 30.1 (6)	294 ± 45.9 (6)	912 ± 336 (6)	17.1 ± 4.8 (6)	108.0 ± 29.9 (6)
III. Tolbutamide	47.6 ± 10.1 (6)	5.33 ± 0.65 (6)	162.6 ± 14.9 (6)	270 ± 106.4 (6)	1194 ± 308 (6)	15.4 ± 6.0 (6)	85.0 ± 21.6 (6)
Set 'B'							
IV. Cholesterol	72.2 ± 16.1 (6)	11.16 ± 0.96 (6)	340.7 ± 67.3 (6)	2154 ± 815 (6)	1174 ± 254 (6)	10.8 ± 2.52 (6)	296.0 ± 97.6 (6)
V. Insulin + Cholesterol	76.5 ± 8.0 (6)	10.6 ± 0.80 (6)	366.8 ± 50.97 (6)	2183 ± 455 (6)	1108 ± 715 (6)	15.0 ± 6.5 (6)	359.0 ± 100.4 (6)
VI. Tolbutamide + Cholesterol	75.4 ± 9.3 (6)	10.97 ± 1.76 (6)	536.0 ± 11.9 (6)	1553 ± 785 (6)	1458 ± 273 (6)	13.3 ± 8.6 (6)	256.0 ± 55.0 (6)

Values are means ± S.D., Figures in the parenthesis indicate number of observations.

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BOOK REVIEW

Title : **PROGRESS IN MOLECULAR
AND SUBCELLULAR BIOLOGY, Vol. 8**

Editors : F. E. Hahn, D. J. Kopecko and
W. E. G. Muller.

36 figures, 147 pages, 1983.

Cloth ISBN 3-540-12590-6

Berlin, Heidelberg, New York, Tokyo ;
Springer-Verlag.

This volume contains some excellent and selected contributions which are recent advances in particular areas of life sciences. The subject matter is very informative and lucidly treated. The first article on "Penicillin until 1957" describes the discovery of penicillin and illustrates how early research observations and experimental findings, often forgotten or no longer read, could bring to light a wealth of information of contemporary interest if pursued with attention. From the discovery of penicillin in 1928 by Fleming, the progress in information on its biological effects and mechanism of action is detailed. The biochemical theory of inhibition of cell wall biosynthesis by penicillin still seems to find favour as the logical explanation of its mechanism of action.

The second chapter in this volume gives an account of the enzymatic repair systems in bacteria and their viruses. It is known that damage to cellular DNA or inhibition of DNA replication induces in *E. coli*, an array of coordinately controlled functions (SOS responses) which effectively repair the conditions. The mecha-

nism of SOS repair functions are lucidly described here.

Information on the trichothecene mycotoxins which are a structurally related group of sesquiterpenoid compounds produced by several genera and species of imperfect fungi, is reviewed in the next article. The subjects treated are trichothecene detection, production, distribution and biological and biochemical actions. In addition, the physiological mechanism of trichothecene toxicity is discussed extensively followed by their metabolism.

In the succeeding article, the current knowledge on the biochemical nature of the scrapie agent, a unique self-replicating pathogen causing infection in sheep and goats, is summarised. Biochemical characterisation has been carried out, which once understood, could lead to the search for the involvement of this type of agent in the etiology of other diseases. New theoretical insights on how these pathogens might replicate is also presented and several experimental methods are offered to test these hypotheses.

Lastly, there is an excellent review on 'Non-cholinesterase effects of anticholinesterases' where evidence is presented that organophosphate compounds (OP's) which are believed to be anticholinesterases, also react with other enzymes (proteases) present in brain and nervous tissue and involved in neuronal function. By such interaction toxic organophosphate compounds could produce undesirable actions in ways other than

through acetylcholine poisoning. The structural characteristics and properties of serum proteases and several currently known neuropeptides are discussed. The view is presented that the various neuropeptides which illustrate peptide homology, could arise by selective proteolytic cleavage of a large molecular weight substance a "master protein" from which these biologically active peptides could owe their evolutionary origin. It is concluded that organophosphates may adversely affect the process of formation and/or degradation of these peptides to account for many of the adverse effects of organophosphate anticholinesterases, which, therefore, cannot be attributed to acetylcholine accumulation alone.

It is altogether refreshing to note that heavy technological details and other procedures of genetic engineering usually associated with molecular biology are not found here. Rather this book offers a multifaceted view of the current progress in molecular biology which would undoubtedly appeal to a wider section of readers.

(Dr. P. Ramakrishnan)
DEPARTMENT OF BIOCHEMISTRY

Title **PROTEINASE INHIBITORS :**
Medical and Biological Aspects.

Editors : Nobuhiko Katunuma,
Hamao Umezawa and Helmut Holzer.
318 pages, 1983 ;
Cloth ISBN 4-6371-0
Berlin, Heidelberg, New York, Tokyo ;
Springer-Verlag.

The role of proteinases in important biological processes like regulation of

intracellular protein metabolism, post-translational processing of precursor proteins and peptide hormones, is well recognised. Disorders of the proteolytic process lead to various pathological phenomena. In view of this, increasing attention is being given to the rapid development and application of proteinase inhibitors of both exogenous and endogenous origin, since such a study would be necessary for an understanding of biological function and disease processes. Further, low molecular weight inhibitors are contributing to the identification of proteases and their functions.

It is hence gratifying that this book has compiled a substantial portion of the recent knowledge gained in the medical and biological aspects of proteinase inhibitors.

There are three sections. In the first section which deals with microbial, plant and synthetic inhibitors, and low action molecular weight protease inhibitors of microbial origin are discussed including their structure and biological action. These inhibitors are believed to bind to the cells and modify immune responses. Hence they exhibit a therapeutic effect against muscular dystrophy and induce changes in the activities of hydrolytic enzymes in the muscles of dystrophic mice. A variety of synthetic inhibitors were tried and found to be specifically active against thiol proteases. Apart from this, synthetic inhibitors have also been used in research on the complement system of blood in various immunological diseases. A detailed study on the amino acid structural make-up and mechanism of inhibition of the trypsin-chymotrypsin inhibitors from peanut (*Arachis hypogaea*), has

brought to light the nature of the reactive sites which exhibit preferential binding of enzyme trypsin over chymotrypsin.

Relating to carcinogenesis, the role of proteinases involved in carcinogen induced sister chromatid exchange (SCE) and mutagenesis of tumor cells, have been studied using microbial proteinase inhibitors. Besides, exemplifying a wider scope of application, the role of exogenous proteinase inhibitors in preventing substrate-unspecific degradation as part of the inflammatory process, is described. This indicates that early administration of suitable inhibitors could probably have a therapeutic effect in humans too.

In the second section of the book, the purification, structure, function and regulatory roles of endogenous thiol proteinase inhibitors are detailed. Experiments are described that discuss the scope and possible use of proteinase inhibitors in experimental systems which may eventually lead to their future use as drugs in cancer therapy.

The third section contains articles on the properties and mode of regulation of

some intracellular proteinases. There is an excellent account of a new approach to the study of the mechanism of intracellular regulation which involves micro-injection of macromolecules into target cells. A whole range of cellular activities from gene expression, assembly and distribution of organellespecific macromolecules and toxin action, to the distribution of proteins could be covered by this approach. A new dimension in cell biology research appears to have been opened up whereby specific macromolecular events can be observed in the presence of numerous other continuous cellular activities.

This book thus offers information on the most recent advances on the medical and biological aspects of proteinase inhibitors and besides, for the first time, we have here an excellently compiled reference book for any one interested in this area of life sciences.

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DEPARTMENT OF BIOCHEMISTRY

DIRECTIONS TO CONTRIBUTORS

Bulletin of Haffkine Institute is a triannual publication. It embodies review and research articles in biomedical sciences, mainly devoted to studies on Communicable Diseases.

Contributions should be precise and concise, highlighting original observations and inferences. The type-script (T.S.) should be sent in duplicate, typed in double space on only one side of the sheet with ample margins.

A brief summary not exceeding 200 words, should accompany the article. The title should be informative and specific, followed by author's name and address.

A short title in not more than forty letters should also be suggested.

The text of the paper should be generally sub-divided into: Introduction, Materials and Methods, or Experimental, Results, Discussion, Acknowledgement and References.

'Short Abstract' of about 100 words suitable for inclusion in 'International Abstracts of Biological Sciences', etc. should be typed on a separate sheet, with author's name, address and title of the paper.

Results should be presented concisely in tables giving only important and relevant data and avoiding incorporation of elaborate observations.

Restraint should be exercised in submitting illustrative matter. Photographs should be well-defined and on glossy paper.

References in the text should be cited by numbers serially as they appear and to be inserted in parenthesis, e.g., 'Plasmid-mediated resistances to these heavy metals have also been observed in enteric bacilli, especially in P factor carrying organisms (1), and have recently been studied by several workers (2, 3, 4)'. References at the end should be arranged numberwise serially as they figure in the text with author's name first and abbreviations according to Index Medicus. The following pattern may be followed:

- (i) Reuben, R.; Carey, D. E. and Meyers, R. M. (1968) — Japanese encephalitis studies in Vellore, S. India, *Indian J. Med. Res*; 56, 109-118.
- (ii) Cruickshank, R. (1968) — Medical Microbiology, 11th Ed. 180-4, E and S Livingstone Ltd., Edinburgh, p. 28.

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