SCIENCE PAPERS

THE STABILITY OF SOLUTIONS OF THE INTERNATIONAL PYROGEN REFERENCE PREPARATION

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The stability of dilute solutions of the International Pyrogen Reference Preparation has been studied. When the response is plotted against log dose a straight line is obtained. Steaming for 30 min. causes a loss of at least 52 per cent of activity and autoclaving at 115° for 30 min. practically complete destruction. Storage in a refrigerator (approximately 4°) for 16 weeks caused a loss of activity. The pyrogen is removed from solution by a strongly basic anion exchange resin and is unaffected by a strongly acidic cation exchange resin.

The desirability of a standard for pyrogens was first discussed at a meeting of the World Health Organisation Expert Committee on Biological Standardisation (W.H.O., 1951), when the problem was outlined by Paton (1950). After a further meeting, held in 1953 (W.H.O., 1953), two pyrogens were made available for study—a partially purified extract from *Proteus vulgaris* and a purified lipopolysaccharide from *Serratia marcescens*. The results of these studies were inconclusive and, at a meeting held in 1956, a highly purified pyrogen from *Shigella dysenteriae* was chosen as a purer and more easily reproducible product (W.H.O., 1959).

The preparation and purification of this material were described by Davies, Morgan and Mosiman (1954) and its properties and method of handling by Humphries and Bangham (1959). Its full description is the "O" somatic antigen of *S. dysenteriae* (shiga) (type I of Kaufmann, 1951), strain K.624 "smooth".

Todd (1955) recommended the adoption of Westphal's lipopolysaccharide from *Salmonella abortus equi* ("Pyrexal") as a standard and has used it as such. He has criticised the choice of the *S. dysenteriae* pyrogen (Todd, 1960).

Since it is obviously desirable to compile as much information as possible on the new reference preparation several studies were undertaken.

Experimental

The International Pyrogen Reference Preparation (IPRP) was obtained as a freeze-dried solid in ampoules each containing 2 mg. A solution was made in water for injection and this was distributed into sterile ampoules to give quantities of 0·25 mg. of IPRP. The solution was then freeze-dried and the ampoules were sealed.

When required, dilutions containing 50 ng. IPRP per ml. in water for injection were prepared from these ampoules on the day of experiment.

An intravenous dose of 1 ml./kg. of this dilution is sufficient to produce a rise of temperature of about 1° in a rabbit.

The animals used were male rabbits of several breeds, weighing between 2 and 4 kg. They had all been injected previously with pyrogens but were not tolerant. They were fed on the National Institute for Medical Research Diet No. 18 with cabbage and drinking water in addition. Food and drink were withheld during tests. Rectal temperatures were measured with a thermistor apparatus (Whittet, 1958). All injections were given into ear veins, in the early afternoon at approximately the same time in each experiment.

TABLE I

EFFECT OF VARIOUS DOSES OF INTERNATIONAL PYROGEN REFERENCE PREPARATION ON THE TEMPERATURES OF RABBITS

Dose ng./kg.	No. of tests	Mean response °	S.D.
50·0	12	1·05	±0·30
10·0	12	0·62	±0·33
2·0	12	0·28	±0·17
0·4	12	0·12	±0·07

TABLE II

EFFECT OF HEAT ON THE STABILITY OF THE INTERNATIONAL PYROGEN REFERENCE PREPARATION

Dose 50 ng./kg.	No. of tests	Mean response °	S.D.
Unheated solution	12	1·02	±0·34
Steamed solution	12	0·49	±0·30
Autoclaved solution	12	0·23	±0·15

TABLE III

EFFECT OF STORAGE ON THE STABILITY OF THE INTERNATIONAL PYROGEN REFERENCE PREPARATION

Dose 50 ng./kg.	No. of tests	Mean response °	S.D.
Fresh solution	12	1·08	0·32
Solution stored 9 weeks at 4°	3	1·16	—
Solution stored 16 weeks at 4°	3	0·58	—

Construction of log-dose-response line. Twelve rabbits and four doses (50, 10, 2 and 0·4 ng./kg. weight) were used.

Three rabbits were each given a dose of each level on four different occasions until each animal had received each dose. The order of administration was decided by means of a Latin square.

The mean responses of 12 rabbits to each dose, together with their standard deviations are shown in Table I.

Effect of heat. Solutions containing 50 ng./ml. of IPRP were treated (*a*) by steaming at 90 to 100° for 30 min., (*b*) by autoclaving at 115° for 30 min. When cool the solutions were injected into rabbits in a dose of 1 ml./kg., unheated samples being used as controls. The results are shown in Table II.

Effect of storage. A solution of IPRP (50 ng./ml.) was tested when freshly made and again after storage at approximately 4° for 9 weeks and

then for 16 weeks. A dose of 50 ng./kg. was used. The results are shown in Table III.

Effect of ion-exchange resins. Solutions containing 50 ng./ml. of IPRP were passed through columns (approx. 15 cm. in length and 3 cm. in diameter) of either Zeocarb 225 (cationic) in the hydrogen form or Deacidite FF (anionic) in the hydroxyl form. The effluents were injected into rabbits in a dose of 1 ml./kg. The results are shown in Table IV.

TABLE IV

EFFECT OF ION-EXCHANGE RESINS ON THE INTERNATIONAL PYROGEN REFERENCE PREPARATION

Dose 50 ng./kg.	No. of tests	Mean response °	S.D.
IPRP solution:			
Untreated	12	0·96	±0·31
Treated with Zeocarb (cation resin) ..	12	0·81	±0·30
Treated with Deacidite FF (anion resin)	12	0·30	±0·37

RESULTS AND DISCUSSION

The theoretical mean response value for each dose used in the construction of the log-dose response line was calculated from the experimental data by means of the equation $Y = \bar{y} + b(x-\bar{x})$. They were 50 ng./kg. = 0·99°; 10 ng./kg. = 0·68°; 2 ng./kg. = 0·36°; 0·4 ng./kg. = 0·07°.

The results show that the preparation gives a log-dose response line in which the practically determined points are close to the calculated best straight line. A dose as low as about 5 ng./kg. is sufficient to give a rise in temperature sufficient to fail the B.P. pyrogen test.

Comparison of the response of unheated IPRP solution with that of steamed solution showed that at least 52 per cent of the activity was destroyed.

The autoclaved solution gave a mean rise in temperature of only 0·30°. Assuming that this is all due to undestroyed pyrogen, autoclaving by the pharmacopoeial process has caused destruction of 70 per cent of the activity. In practice, however, a mean response of about 0·3° is not uncommon in a negative pyrogen test and the B.P. allows a mean rise of 0·33° in a test using 3 rabbits and of 0·55° in a test using 12 rabbits before a solution fails the test.

The mean response in 12 rabbits of the steamed solution is 0·49° and that of the autoclaved solution 0·30° and thus both would pass the B.P. pyrogen test.

Whittet (1961) found a mean response of 0·21° in 100 pyrogen tests on products made with every precaution to avoid pyrogens and believed to be pyrogen-free.

The responses of the 12 tests on autoclaved solution were compared with those of 12 tests on solutions believed to be pyrogen-free (mean response 0·20°) by means of the *t* test and were found not to be significantly different ($t = 0{\cdot}0924$; $P = >0{\cdot}9$; D.F. = 22). The difference in response between the unheated and autoclaved solutions was highly significant ($t = 7{\cdot}999$; $P = <0{\cdot}001$; D.F. = 22). The difference in response

between the unheated and steamed solutions was also highly significant ($t = 8{\cdot}135$; P = <0·001; D.F. = 22).

In the storage tests no significant destruction had occurred in 9 weeks, but by 16 weeks almost half the activity had been lost.

The tests with ion-exchange resins showed that IPRP is probably completely adsorbed by the strongly basic anion exchange resin Deacidite FF in the hydroxyl form and is unaffected by the strongly acidic cation exchange resin Zeocarb 225 in the hydrogen form. The difference between the responses of untreated solution and that treated by Deacidite FF is highly significant ($t = 5{\cdot}013$; P = <0·001; D.F. = 22). The difference between untreated and Zeocarb treated solution is not significant ($t = 0{\cdot}8810$; P = 0·4 to 0·3; D.F. = 22).

With the exception of its reaction towards heat, the International Pyrogen Reference Preparation has the properties usually attributed to bacterial pyrogens, especially those from Gram-negative organisms. Thus, if the response is plotted against log-dose, a straight line is obtained. After intravenous injection of a suitable dose into rabbits a typical fever curve is obtained after the usual latent period of 20 to 30 min. With high doses a secondary rise in temperature occurs.

The effect of ion-exchange resins on IPRP resembles that on the pyrogens of *P. vulgaris*, *S. abortus equi* and *Pseudomonas aeruginosa* (Whittet, 1956, 1958).

Since the early reports by Seibert (1923) and those of Banks (1934), which dealt with only two pyrogens from named organisms (*Pseudomonas ureae* and *Pseudomonas scissa*), there has been little systematic work on the stability of pyrogens.

On the basis of their work, it appears to have been accepted that all pyrogens are extremely thermostable. Wylie and Todd (1948, 1949) examined the effect of autoclaving at 120° on pyrogens from *P. vulgaris*, *Bacillus subtilis*, *Ps. aeruginosa* and *Micrococcus tetragenes*. The first three lost 50 per cent of their activity in 30 min. and 95 per cent in 2 hr. *M. tetragenes* pyrogen, however, lost only 20 per cent of its activity in 2 hr. They concluded that the pharmacopoeial autoclaving process would have little effect on a dilute solution of pyrogen such as might arise from accidental contamination of water and that sterilisation is of no practical value in removing pyrogen from solutions intended for parenteral administration. Whittet (1958, 1961) showed that the pyrogenic activity of London tap water is completely destroyed by autoclaving at 115° for 30 min.

There is much variation in the stability of pyrogens from different sources and, now that numerous different purified preparations are available, further stability studies should be made.

REFERENCES

Banks, H. M. (1934). *Amer. J. Clin. Path.*, **4**, 260–291.
Davies, D. A. L., Morgan, W. T. J. and Mosiman, W. (1954). *Biochem. J.*, **56**, 572–581.
Humphries, J. H. and Bangham, D. R. (1959). *Bull. Wld Hlth Org.*, **20**, 1241–1244.
Paton, W. D. M. (1950). *Wld Hlth Org. Tec. Rep. Ser.*, 36.
Seibert, F. B. (1923). *Amer. J. Physiol.*, **67**, 90–104.
Todd, J. P. (1955). *J. Pharm. Pharmacol.*, **7**, 625–641.

Todd, J. P. (1960). *Pharm. J.*, **2**, 53–55.
Whittet, T. D. (1956). *J. Pharm. Pharmacol.*, **8**, 1034–1041.
Whittet, T. D. (1958). *Experiments on the Occurrence of Pyrogens in Water and their Removal and on their Pharmacology and Fate in the Body.* Ph.D. Thesis, Lond. Univ.
Whittet, T. D. (1961). *Public Pharmacist*, **18**, 18–23.
World Health Organisation, Expert Committee on Biological Standardisation
(1951). *Wld Hlth Org. Tech. Rep. Ser.*, 36.
(1953). *Wld Hlth Org. Tech. Rep. Ser.*, 68.
(1960). *Wld Hlth Org. Chronicle*, **13**, 443–444.
Wylie, D. W. and Todd, J. P. (1948). *Quart. J. Pharm. Pharmacol.*, **21**, 240–252.
Wylie, D. W. and Todd, J. P. (1949). *J. Pharm. Pharmacol.*, 1949, **1**, 818–835.

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