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Studies with the International Pyrogen Standard on the sensitivity and reproducibility of pharmacopoeial pyrogen testing

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Rabbits, 27 or 36 in each experiment, were injected with the International Pyrogen Standard (I.P.St.) in different seasons. The maximum temperature rises were registered, randomized and interpreted according to the requirements of the B.P. (1973), U.S.P. (1970), P. Hung. (1970) and P. Nord. (1962). Although the dose of 3·5 ng kg^{-1} I.P.St. proved to be non-pyrogenic as tested in summer, when tested in winter the same dose was qualified pyrogenic (to be rejected) by up to one third of the combinations by the criteria of the four Pharmacopoeias. In the spring experiment "to be rejected" qualifications predominated as based on the response of large groups of rabbits. Exclusion of the rabbits showing low sensitivity (before randomization) barely influenced the results with 3·5 ng kg^{-1} I.P.St. in the experiment in which the mean temperature rise was 0·49°. If, however, the mean temperature rise was higher (0·57 or 0·69°), such a selection practically resulted in the disappearance of "passable" qualifications in the triplet groups and a great predominance of "to be rejected" qualifications in the larger groups. The dose 7·0 ng kg^{-1} consistently proved to be pyrogenic in large groups of rabbits.

In the summer of 1971, 36 rabbits were given 3·5, 7·0 and 14·0 ng kg^{-1} I.P.St. intravenously, at intervals of 2 to 3 weeks. The experiment was repeated in January, 1972, with another set of 36 rabbits and in the spring 1972 on 27 rabbits. The results were analysed according to the requirements of the B.P. (1973), U.S.P. (1970), P. Hung. (1970) and P. Nord. (1962). In the first cycle of the analysis all the rabbits were allocated and seasonal differences were studied. In the second cycle the rabbits of low sensitivity were excluded from analysis.

MATERIALS AND METHODS

The ampoule kindly supplied by Dr D. R. Bangham contained 2 mg I.P.St. in the lyophilized state. This was dissolved in 10·0 ml pyrogen-free distilled water, distributed in 0·1 mg aliquots in ampoules and relyophilized. Ampoules were opened on the day of use and the content dissolved in 0·9% (w/v) pyrogen-free saline to obtain the necessary dilution. The rabbits were injected intravenously. All the animals, brown Hungarian "vadas" rabbits of either sex, 1·8 to 4·0 kg, were purchased from the same breeder and kept for at least one week before the experiment in individual cages at room temperature on rabbit feed dragée and water. The animals grew in weight as required. In experiments only those rabbits were included whose initial rectal temperature ranged between 38·0 and 39·8°. Also, those whose response to 10

ml kg^{-1} pyrogen-free isotonic saline did not fall within the requirements of the B.P. were excluded.

On the eve of the experiment the rabbits were placed into the individual cages of the test laboratory and all food withheld until the end of the experiment. Water was allowed until the start of experiment. In the morning of the experiment the rabbits were placed in test stocks and their necks were fixed. The applicator of a thermocouple (Elektro Laboratoriet, Copenhagen) was placed in the rectum and fixed to the tail. Readings were taken 90 and 120 min thereafter. The second value was regarded as initial temperature provided the difference between the two temperature values did not exceed 0·2°. Within a few min after the second measurement each rabbit was intravenously injected with I.P.St. at room temperature. The volume injected was 1·4 ml kg^{-1} or less. Subsequently the rectal temperature of the rabbits was controlled at 30 min intervals over 3 h. The difference between maximum and initial temperature for each rabbit was taken as its response.

The responses of the rabbits in each experiment were analysed according to the four Pharmacopoeias. The results from the rabbits were randomly distributed by the aid of numbered cards into groups consisting of 3, 6, 9 and 12 animals as prescribed in B.P., 3 and 8 animals (U.S.P.), 3, 6 and 9 animals (P. Hung.) and 5 animals (P. Nord.). In this way 9 or 12 groups consisting of 3 rabbits, 4 or 6 groups consisting of 6 rabbits, 4 or 6 groups consisting of 5 rabbits, 3 or 4 groups consisting of 8 or 9 rabbits and 2 or 3 groups of 12 rabbits were established from the 27 and 36 animals respectively.

To increase the combinations in number, 108 groups consisting of 3 rabbits each at a time ($_{36}C_3$) were further randomized using numbered cards. From these were pooled by further randomization 54 groups of 5 rabbits each, one was randomly omitted from those of 6. Thirty-six groups of 8 rabbits were obtained similarly from groups of 9. Qualification was according to the respective pharmacopoeial requirements.

RESULTS

Table 1 shows the mean responses in different seasons. The straight lines and the equations were computed according to Burn, Finney & Goodwin (1950). Doubling of the pyrogen doses consistently resulted in distinct differences in the mean response. That to 3·5 ng kg^{-1} I.P.St. was significantly lower in summer compared to the spring ($P<0·01$) and winter ($P<0·05$) values. The regression coefficient was highest for the summer and significantly different when compared to that for either of the other seasons.

Table 1. *Regression lines for the International Pyrogen Standard.*

Day of experiment	I.P.St. Dose ng kg^{-1}	Mean temperature rise °C	Regression line
27/8/71	3·5	0·286	
4/7/71	7·0	0·691	$y = 0·656 + 1·214$
15/7/71	14·0	1·016	$(x-0·845)$
5/1/72	3·5	0·487	
22/12/71	7·0	0·887	$y = 0·775 + 0·8081$
10/1/72	14·0	0·963	$(x-0·845)$
30/3/72	3·5	0·563	$y = 0·775 + 0·8081$
18/4/72	7·0	0·766	$(x-0·845)$

In Table 2 the pyrogenicity qualifications for 3·5 ng kg^{-1} I.P.St. as obtained from the summer, winter and spring experiments are presented. In summer according to the B.P. and P. Hung. no group was classified "to be rejected", though for the triplets a number were classified "to be retested". According to the U.S.P. from the 36 groups of 8, 34 yielded "passable" and 2 yielded "to be rejected". From the groups of 5 (P. Nord.) 49 were scored "passable" and 5 were scored "to be rejected".

In contrast to the summer experiment in which the 3·5 ng kg^{-1} dose proved to be practically non-pyrogenic, in the winter experiment "to be rejected" qualifications were many. The winter qualification was "to be rejected" in 7 (25·9%) of the 27 B.P. groups of 12 and the respective results for the 36 U.S.P. groups of 8 were 14 (38·8%), for the 36 P. Hung. groups of 9, 9 (25·0%) and for the 54 P. Nord. groups of 5, 18 (33·3%). In the spring experiment most of the large groups showed the 3·5 ng kg^{-1} dose "to be rejected". According to the B.P. the "to be rejected" qualifications applied to 17 groups (62·9%). The respective numbers and % for the U.S.P. were 31 (86·1%), for the P. Hung. 29 (80·5%), and for the P.Nord. 44 (81·4%).

Table 2. *Qualification of 3·5 ng kg^{-1} International Pyrogen Standard according to five Pharmacopoeias.*

Pharmacopoeia	No. of rabbits per group	Number of groups	Number of groups yielding "passable"			"to be rejected"			"to be retested"		
			a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
B.P. and Europ.P.	3	108	75	31	13	0	1	3	33	76	92
	6	54	53	26	11	0	0	1	1	28	42
	9	36	36	24	5	0	0	3	0	12	28
	12	27	27	20	10	0	7	17	0	0	0
U.S.P.	3	108	75	45	14	0	0	0	33	63	94
	8	36	34	22	5	2	14	31	0	0	0
P. Hung.	3	108	71	35	13	0	1	3	37	72	92
	6	54	50	26	7	0	0	1	4	28	46
	9	36	36	27	7	0	9	29	0	0	0
P. Nord.	5	54	49	36	10	5	18	44	0	0	0

a 36 rabbits were given 3·5 ng kg^{-1} I.P. St. on 27 August 1971. Mean temperature rise 0·286°.
b 36 rabbits were given 3·5 ng kg^{-1} I.P. St. on 5 January 1972. Mean temperature rise 0·478°.
c 27 rabbits were given 3·5 ng kg^{-1} I.P. St. on 30 March 1972. Mean temperature rise 0·574°.

The results with 7·0 ng kg^{-1} I.P.St. proved this dose to be pyrogenic in nearly all the large groups irrespective of the season. Therefore the results are not included.

Taking the groups of three rabbits in the winter experiment into account, the numbers (and percentages) of the "passable" qualifications were as follows: B.P. 31 (28·7%); U.S.P., 45 (41·6%); P. Hung., 35 (32·4%). The respective figures from the spring experiments were: B.P., 13 (12·0%), U.S.P., 14 (12·9%), P. Hung., 13 (12·0%).

In the larger groups the % "to be rejected" was the highest according to the U.S.P. in both the winter and spring experiments. In the winter this percentage was least according to the B.P. and the P. Hung. In the spring the B.P. scores gave the lowest percentage "to be rejected" while the P. Hung. and P. Nord. values were slightly higher than those given by the U.S.P.

With the $_{36}C_3$ combinations in the winter, the U.S.P. gave the highest and the B.P. the lowest passable values with the P. Hung. value slightly higher than that given by the B.P. In the spring there were practically no differences.

Exclusion of the rabbits of low sensitivity

In this re-examination of the data the rabbits responding to 14 ng kg^{-1} I.P.St. with a temperature rise less than 0·75° and/or to 7·0 ng kg^{-1} with less than 0·6° were excluded from the further analysis. The remaining rabbits were randomized as described.

The scores obtained after the exclusion of less sensitive animals are presented in Table 3. They are thus to be compared with those parts of Table 2 which represent the results of experiments done in winter (Table 2*b*) and spring (in Table 2*c*). It can be seen that although in all groups while the number of "passable" qualifications fell the number and % of "to be rejected" scores increased. The exclusion of the rabbits giving a feeble response was of more decisive effect on the score for 3·5 ng kg^{-1} I.P.St. in the spring than in the winter experiment. In winter numerous groups of the selected rabbits gave "passable" whereas in spring "passable" almost disappeared.

The 7·0 ng kg^{-1} I.P.St. proved to be pyrogenic or "to be retested" in almost every combination even without selection so the selection could cause little change.

Table 3. *Qualification of 5·4 ng kg^{-1} International Pyrogen Standard according to five Pharmacopoeias after exclusion of rabbits showing low sensitivity.*

Pharmacopoeia	No. of rabbits per group	Number of groups	Number of groups yielding "passable"		"to be rejected"		"to be retested"	
			b	*c*	*b*	*c*	*b*	*c*
B.P. and Europ.P.	3	108	19	0	2	8	87	100
	6	54	10	0	0	17	44	37
	9	36	10	0	2	22	24	14
	12	27	14	0	13	27	0	0
U.S.P.	3	108	23	2	0	0	85	106
	8	36	10	0	26	0	0	0
P. Hung.	3	108	17	1	2	8	89	99
	6	54	4	0	0	14	50	40
	9	36	10	0	26	36	0	0
P. Nord.	5	54	19	4	35	50	0	0

b From the 36 rabbits given 3·5 ng kg^{-1} I.P. St. on 5 January, 1972 (see Table 2), 13 rabbits of low sensitivity were excluded, from the remaining 23, combinations were randomized. Mean temperature rise 0·523°.

c From the 27 rabbits given 3·5 ng kg^{-1} I.P. St. on 30 March, 1972 (see Table 1), 8 rabbits of low sensitivity were excluded, from the remaining 19, combinations were randomized. Mean temperature rise 0·684°.

DISCUSSION

The present experimental study was undertaken first of all to estimate (*i*) the risk of contradictions and mis-qualifications in the pyrogen tests performed in strict adherence to the Pharmacopoeias cited; (*ii*) how qualifications are influenced by the exclusion of the rabbits of low sensitivity; (*iii*) to compare the qualifications of the four Pharmacopoeias under like conditions.

In the series of all the rabbits the summer responses to 3·5 ng kg^{-1} I.P.St. rarely yielded as "to be rejected" qualification save a few instances in the U.S.P. large groups and the P. Nord. group 5.

In the winter experiment the mean response was higher, consequently the score "to be rejected" was augmented. Nevertheless, most groups indicated lack of pyrogenicity. In the spring experiment the mean response to the dose 3·5 ng kg^{-1} was still higher, consequently, this dose failed to pass the test in most larger-size test

groups. The response was significantly lower in summer than in spring ($P<0{\cdot}001$) or winter ($P<0{\cdot}05$).

All the experiments were performed in the morning. The mean weights for the rabbits receiving 3·5 ng kg^{-1} in the summer winter and spring experiments were not statistically significant being $3143 \pm 60{\cdot}3$ g, $3283 \pm 101{\cdot}8$ g and $3030 \pm 72{\cdot}6$ g, respectively. The respective room temperatures were 23, 20 and 22°.

It is well-known that endotoxins are highly immunogenic (see Landy, 1971). It is therefore possible that the temperature rise of the rabbits was influenced by re-use. We obtained the highest response to 3·5 ng kg^{-1} in spring, when this dose was the first stimulus for the rabbits in use. On the other hand, the lowest rise was induced in the summer, when this dose was administered after the other two doses.

The 7·0 ng kg^{-1} I.P.St. dose proved to be pyrogenic in all but a few cases. In the summer, 14·0 ng kg^{-1} I.P.St. induced a mean response of 1·02° with lowest values 0·3, 0·5 and 0·7°. This dose will invariably fail the test performed, according to B.P., U.S.P. and P. Hung. on 3 rabbits or according to P. Nord. on 5.

According to the French, GDR and Rumanian Pharmacopoeias, rabbits should be injected with a pyrogen standard material before pyrogenicity tests, and those of low sensitivity should be excluded. In England, Todd (1960) widely applied the pyrogen reference preparations, for such purposes. The same has been done by the Farbwerke Hoechst (Hergott & Klavehn, 1971). According to the GDR and the Rumanian Pharmacopoeias, Pyrogen standard should be injected in an amount that induces an approximately 1° rise in the body temperature. The rabbits responding with a rise of <0·75° (Rumanian P.) or <0·8° (GDR) are insufficiently sensitive. We wished to check the efficiency of such a selection of rabbits.

In our experiments exclusion of the rabbits of low sensitivity influenced the score depending on the mean response for the corresponding experiment. In an experiment with a mean temperature rise of 0·49° the selection increased the number of "to be rejected" qualifications and reduced the "passable" ones, yet, the qualification in general was but little influenced. On the other hand, in the experiments in which the mean maximum rise was as high as 0·57 or 0·69° without selection, after selection the number of "passable" qualifications on 3 rabbits was much reduced or disappeared and the responses "to be rejected" much increased in the relatively large groups.

Repeated administration of endotoxins may lead to a non-specific tolerance. The endotoxin of a given serotype may induce cross tolerance to serologically unrelated endotoxins (Work, 1971). It is thus conceivable that the low sensitivity observable in certain rabbits when pretreated with pyrogen standard will be observable with heterogeneous pyrogen materials as well. Since in our investigations the probability of qualifying a pyrogenic preparation as "passable" was reduced by preliminary test with the I.P.St. and the exclusion of the insufficiently sensitive rabbits, this procedure seems to be useful in increasing of the adequacy of the pyrogen test.

It should however be noted that the injection preparations produced in Hungary have been controlled for pyrogenicity by our Institute for 16 years. The preparations under testing have ranged between 5000 and 10 000. In this period the rabbits were not subjected to a sensitivity test and thus those of low sensitivity were not excluded. In spite of this, a preparation had to be withdrawn because of pyrogenicity during its clinical use less than 10 times.

The differences in pyrogenicity score are partly explainable by the fact that in the different Pharmacopoeias the limit of the total temperature rise is given at more or

less different levels. For example, when calculated with groups of three rabbits, a total temperature rise of 1·15° and 1·20° is the limit of a "passable" qualification according to the B.P. and the P. Hung., respectively. According to the U.S.P. the corresponding limit is at 1·4°. Consequently, in the winter experiment the "passable" qualification was the highest as calculated according to the U.S.P. (41·6%), compared to the B.P. (28·7%) and P. Hung. (32·4 %). The differences were still larger when the "to be rejected" qualifications were calculated from larger rabbit groups.

In the B.P. the limit for 12 rabbits is given as 6·6°, in the U.S.P. that for 8 rabbits is given as 3·7°. Calculating the latter value for 12 rabbits would result in 5·55°, i.e. 1·05° lower than the limit prescribed by the B.P. It is therefore clear why the highest percentage of "to be rejected" qualification was obtained, in both the winter and spring experiment, when calculated according to the U.S.P. (38·8% and 86·1%). The respective values as calculated according to the B.P. were 25·9 and 62·9%.

The Hungarian brown "vadas" rabbits used developed a considerable response to 3·5 ng kg^{-1} I.P.St., like the rabbits used by other investigators to 3·0 ng kg^{-1}I.P.St. (Humphrey & Bangham, 1959; Bangham, 1971).

The pyrogenic effect of various doses of I.P.St. have been investigated by Palmer & Whittet (1961) and Chatterjee & Das (1969). For the data published by these authors we have computed the equations as follows: $Y = 0{\cdot}525 + 0{\cdot}411\ (x - 0{\cdot}649)$ Palmer & Whittet (1961); $Y = 0{\cdot}523 + 0{\cdot}569\ (x - 0{\cdot}965)$ Chatterjee & Das (1969).

Our regression lines (Table 1) are steeper than those given by these equations, suggesting that our rabbits were more sensitive.

We have applied the tests prescribed in the P. Hung. and those in P. Nord. in addition to the B.P. and U.S.P. tests. In P. Hung. tests the advantages of the B.P. and U.S.P. requirements are combined, whereas the test in the P. Nord is the simplest of all the pharmacopoeial pyrogen tests known by us.

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REFERENCES

BANGHAM, D. R. (1971). Ciba Foundation Symp. *Pyrogens and Fever.* pp. 207–214. London: A Churchill.

British Pharmacopoeia (1973). Appendix XIV. J.A. 115 London: Her Majesty's Stationery Office.

BURN, J. R., FINNEY, D. J. & GOODWIN, L. G. (1950). Biological Standardization. 51–59. London: Oxford Univ. Press.

CHATTERJEE, K. P. & DAS, I. (1969). *Experientia*, **25**, 52.

Deutsches Arzneibuch (GDR) 7. (1964). Vol. I. Berlin: Akademia.

European Pharmacopoeia (1971). Vol. II. 58. Sainte Ruffine: Maisonneuve.

Farmacopoea Romana (1965). A. VIII–A. Bucuresti: Edit. Medicala.

HERGOTT, J. & KLAVEHN, J. (1971). *Arzneimittel Forsch.*, **21**, 553–559.

HUMPHREY, J. H. & BANGHAM, D. R. (1959). *Wld. Hlth. Org.*, **20**, 1241–1244.

VI. Hungarian Pharmacopoea (1970) English edn. Vol. I. 168–169 Budapest, Akadémiai Kiadó.

LANDY, M., See Bangham: pp. 49–56.

PALMER, C. H. R. & WHITTET, T. D. (1961), *J. Pharm. Pharmac.*, **13**, Suppl. 62T–66T.

Pharmacopée Francainse (1965). VIII. Paris: Comission pharmacopée.

Pharmacopoea Nord. (1962). Editio Danica Vol. IV. 29. Kobenhaven: Nyt Nordisk.

TODD, J. P. (1960). *Pharm. J.*, **178**, 53–55.

United States Pharmacopeia (1970). 18. Revision. 886. Bethesda; U.S.P. Convention.

WORK, E. (1971). See: Bangham: pp. 23–47.