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The removal of pyrogen substances from injection solutions through filtration.

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Preparation of pyrogen-free injection solutions is becoming increasingly important from year to year because new preparations are continuously being marketed for intravenous application, or old preparations hitherto used in a form suitable for intramuscular injection are converted by chemical reaction into a water-soluble form suitable for intravenous injection. In this respect mention may be made of steroid hormones difficultly soluble in water which by glucosidising (Hagedorn, Johannessohn, Rabald and Voss 1940 (1), Rabald and Dietrich 1939 (2), and others) but also in their conjugated form, become soluble in water to such a degree that they can be injected intravenously.

With increasing application of intravenous injection the responsibility of the manufacturer of these solutions also grows and he will of course endeavour by all possible means to guarantee absolute compatibility of his preparations, also in intravenous injection. Besides sterility, proper pH-values, etc., freedom of the solutions from pyrogenic substances is one of the main postulations for such preparations. Contamination of the solution with pyrogens (by which are understood both the pyrogenic germs themselves as also their pyrogenic products of metabolism) can be brought about in 5 different ways:

1. The effective substance itself brought into solution can contain such bodies (Co Tui and Wrigth 1942 (3).
2. The solvent, i.e. mostly distilled water, can contain pyrogens (Hart and Penfold 1911 (4)).
3. The ampules or bottles into which the preparation is filled are not pyrogen-free (Sager 1950 (5)).
4. The instruments used for injection, the tubules or injection syringes are not pyrogen-free (Sager 1950 (5)).
5. When puncturing not sufficiently clean skin, pyrogenic germs penetrate the blood channels of the patient and lead to the known fever phenomena.

Of these 5 possibilities the fourth and fifth are outside control of the injection solution manufacturer but are nevertheless just as important as danger sources. All the more reason, however, that he should devote himself assiduously to the three first-named and most frequently occurring sources and endeavour by strictest manufacturing specifications to obtain 100 % safety. Points 1 and 3 are beyond the scope of this article; their observation, especially of the third (freedom from pyrogens of the ampullae and flasks), should not be difficult.

Our investigations were concerned with point 2): freedom of the solvents from pyrogenic substances, and they were focussed not so much on the question of producing an 'a priori' pyrogen-free solvent (Wilke 1953 (6))as on the methods for freeing a solution containing pyrogens from the pyrogenic substances. Two basically different methods are available for this purpose; one aims at destroying the pyrogenic substances and preventing them from becoming effective, the other aims at eliminating the pyrogenic substances. An example for the first-named method is treatment of the solution with hydrogen peroxide. It was suggested by Campbell and Cherkin (1945 (7)) and found to be satisfactory by Taub and Hart (1948 (8) and tested by Menczel (1951 (9)). Charonnat and Lechat (1951 (10)) found it to be ineffective and thought that these contradictory results were attributable to differences in pyrogens. They recommended a method of their own which they had found in practice to be very effective: they added to the solution measured quantities of Eau de Javal, allowed it to act for 30 minutes and then shook with alcohol.

Oxidation as a means for removing pyrogens from finished injection preparations is, however, in most cases out of the question on account of attack on the effective substance. Also, treatment with activated charcoal can only be contemplated for preparing pyrogen-free water and only in very rare cases for finished injection solutions.

The pyrogenic substances can be removed from injection preparations very simply and without affecting their qualities by filtration. This can be stated with absolute certainty for preparation of pyrogen-free water (H. Wilke 1953 (6)), but otherwise on the basis of our own experiments for the time being only with respect to the pyrogen-effective model substances used by us. But in practice experience gained with different solutions has shown that the effective scope of the adsorption filtering process described here after all seems to be very wide. The fact that the method also has its limitations due to physical-chemical composition of the solution will be explained later.

Reports from practice that after sterilising filtration of injection preparations with Seitz sterilising sheets the formerly frequent fever reactions after intravenous application no longer occurred, prompted us about two years ago to begin with series of experiments for explaining these phenomena. A study of the relevant literature disclosed the fact that Co Tui and his assistants had already in 1936 (11) pointed to the effectiveness of Seitz sheets for pyrogen removal. These and all other publications of recent years (Ulmann has given a comprehensive summary in this journal (1953 (12)) were chiefly concerned with the nature of the pyrogens, their origin and action, but removal of pyrogens by filtration is only treated in a few publications purely qualitatively.

Quantitative investigations were indispensable for further development of pyrogen-active filtering methods, and above all for determining their limitations; they should also if possible throw light on the action mechanism.

1. The Test Solutions

When we started experimenting in 1951, the test solution we had at our disposal was a commercial pyrogenic preparation which according to the manufacturer's statement was prepared from coli-bacteria and standardised in experiments with rabbits. The pyrogen content was given in units having a certain relation to the number of germs contained in the different concentrations, one unit corresponding to a content of one million germs. This commercial preparation (we are indebted to Asta-Werke AG., Brackwede for placing the preparation at our disposal) was brought to the concentration required for the test by diluting with pyrogen-free distilled water and was examined before and after filtering in the pyrogen test. After the first test we already came across a remarkable disparity of results which could not be due to the testing technique; neither could heterogeneity of the test animal material be the reason because all rabbits used for the experiments were tested again and again for normal capability of reaction, especially in view of the fact that the French research scientist Dorsche and his colaborators (1950 (13)) had pointed to occurrence of hypo-sensitive and hyper-sensitive animals. It was then found that the commercial preparation in question only contained 10 % pyrogens in solution while the other 90 % of the pyrogenic effectiveness were bound to presence of destroyed bacteria bodies. The experiments with this pyrogenic preparation therefore disclosed comparatively little with regard to the possibilities for removal of dissolved pyrogens by filtration since the main part is in any case retained by the filter media impervious to germs under discussion here. On the other hand, the tests conform fully with the conditions encountered in practice. Here too, the bacterial-pyrogens will be effective in the same proportions in the free state and bound to bacteria bodies, either that solutions containing bacteria are sterilised directly by heat without preceding sterilising filtration as is still the usual procedure with any commercial preparations applied intravenously, or that the living bacteria are caught by a sterilising filtration without subsequent heat sterilisation so that only the already poured out pyrogenic substances have to be retained in the filter or eliminated in some other way.

To obtain quantitative data we continued our experiments with a preparation in which the bacteria protein had been broken up by fermentation so that according to the manufacturer's statements all pyrogenically active substances were present in solution. This preparation was dissolved - in the concentration required for the test - in a physiological salt solution, passed through the filter test, and then evaluated in the rabbit experiment. Parallel to this, controlling tests were invariably carried out in a dilution series with the unfiltered original solution to establish the pyrogenic limit dose. The "units" in which the pyrogen quantities are given in our test results were determined by the manufacturer in accordance with the preparation first used, and they therefore exclusively apply to this preparation. This original preparation was available in solid form, 1 gamma being the equivalent of $\frac{20}{8}$ of the here mentioned units so that 1 unit corresponds to $5 \cdot 10^{-8}$ grammes substance.

2. Pyrogen Testing Technique

In our pyrogen testing technique we have kept to the specifications suggested by one of us (Vo.) at the time (1950 (14)) on the basis of literature and years of experience. These specifications have proved their suitability for pyrogen testing of very different injection solutions as encountered in manufacture on a commercial scale. Here is a short description of the technique:

Experimental animals: rabbits of 2 kg and more, but not over 4 kg; 3 animals per experiment. Temperature check during the 24 hours before beginning the experiment. Final temperature measurement 1 hour before injecting test solution. The temperature then measured is regarded as normal temperature on the basis of which the temperature rise is calculated. On the day of the experiment itself the animals are not fed before injection or during that day. The temperature was measured with a thermocouple feller whose 3 mm thick end was inserted in the rectum to a depth of 7 1/2 cm. The test solution was injected intravenously into one of the marginal veins of the ear; the volume injected varies according to the experiment and according to the calculated or estimated pyrogen content. 45 minutes after injection the temperature is taken for the first time and then every 45 minutes until after 3 hours 4 measurements are available. The experiment is regarded as pyrogenpositive if 2 or all 3 animals show a temperature rise of 0.6°C. or more in excess of normal temperature. If only one of the animals reacts positively the experimentis repeated, if possible, i.e. if sufficient test solution is left, with a batch of 3 fresh animals, and the whole experiment is only regarded as positive if two or all three animals of the fresh batch show positive reaction.

3. Relation Between Pyrogen Quantity and Temperature Rise

From the first, it seemed desirable to arrange our tests for elimination of pyrogenic substances by filtering in such a way that the results of filtration or adsorption were determined quantitively and not confined to the simple statement: "containing pyrogen" or "pyrogen-free".

Difficulties, however, arose primarily on account of the uncertainty as regards the chemical nature of pyrogens. A number of suggestions have been made for "units" of pyrogenic substances but their use is in our opinion impracticable because we can never be certain whether the suggested reference substance, i.e. the standard pyrogen, is identical or not with the unknown pyrogens to be tested. This makes it quite uncertain whether the unknown pyrogens will behave in the rabbit experiment in the same way as the standard pyrogen whose units we intend to use for reference. The excellent and comprehensive test carried out by Tennent and Ott (1952 (15)) which aimed particularly at quantitive evaluation of pyrogens in the animal experiments, also carry this factor of uncertainty in them. Mathematical formulation of the results cannot be of any help because the French research workers Charonnat and Lechat rightly say that the prerequisite for a mathematical formulation, i.e. existence of a linear relationship between temperature rise and logarithm of the pyrogenic dose, is only conditional, or at least not valid for all pyrogens. We find, for example, the series listed in table 1 for pure colipyrogen in true or coloidal solution.

We see that doses 1 and 4, or 2 and 5 respectively, are equal in action although the ratio of the pyrogen quantities is 8 to 1. This makes it probable that the mentioned logarithmic ralation can only be valid for a very limited range of small doses and that it loses its validity as soon as a certain limit dose is reached or, exceeded. A further example is given in table 2.

Table 1

Dose No.	Pyrogen quantity injected U/kg	Temperature rise (mean value of 3 rabbits)
1	30	1,2°
2	15	1,0°
3	7,5	1,5°
4	3,75	1,2°
5	1,875	1,0°

Table 2

Dose No.	Pyrogen quantity injected U/kg	Temperature rise (mean value of 3 rabbits)
6	2	1,5°
7	0,2	0,7°
8	0,1	0,9°
9	0,067	0,7°
10	0,03	0,4°
11	0,04	0,1°

From 30 U/kg down to a dose of 0.1 U/kg (dose ratio 300:1) we again and again obtained practically the same temperature rise varying between 0.9 and 1.5°C., the high and the low values of this interval being spread quite irregularly over the large and small doses (the temperature rise of only 0.7°C., for 0,2 Ukg is specially erratic). Only for the interval from dose 11-8 (dose interval 1:2.5) can a certain regularity of the rise of temperature corresponding to the increasing pyrogen dose be spoken of.

There are, however, indications that a significant temperature rise is also possible after exceeding the mentioned minimum or limit dose, i.e., when the pyrogen doses are excessively increased. Thus Charonnat and Lechat, for example, found the following sequence:

Mill.germs per Kg:	12,5	25	50	100	200	800
Temp. rise:	1,4°	1,3°	2,0°	2,2°	1,8°	2,4°

We see that double the pyrogen dose has no effect but four times the dose is sufficient to bring about a temperature rise, and when increasing the pyrogen quantity 64 times the rise is very marked.

It is surprising how small the quantities are which lead to a pyrogenic raction in rabbits. From table 2 we find by interpolation that for a temperature rise of 0.6°C., which is regarded as the limit for positive pyrogen raction, about 0.06 units per kg per animal have to be injected. According to the above-mentioned relation between substance quantity and unit this corresponds to a quantity of $3 \cdot 10^{-9}$ grammes pyrogenic substance.

Under the described circumstances we are of the opinion that all quantitative statements on pyrogens and also on their elimination can at present only be of a relative nature and are only valid for the pyrogenic preparation just being used and cannot be applied unconditionally to other pyrogens.

4. Filtering Test

General Effect of Sterilising Filtering Media.

After the first investigations leading to clarification of formation and action of pyrogenic substances, these were regarded as filterable, i.e. not capable of being eliminated by filters. However, in the tests described by Hart and Penfold (4) a Berkefeld filter cartridge impervious to germs was used as filtering medium with which - as will be explained in the following - no other result could be obtained. Since practical experience has in the meantime shown that under certain conditions not only germ-free but also pyrogen-free solutions could be obtained with Seitz filter sheets, it was obvious that this must be due to special properties of the filtering material. Varying pore diameters of the different filter media impervious to germs could not explain the varying behavious of the different filtering media because the diameters are of the same order of magnitude for all filtering media impervious to germs. For the sterilising filters tested within the scope of this work the following pore diameters were determined by physical methods:

Berkefeld filter cartridge W	3,5 u.
Fritted glass, Schott G5	1,0 - 1,7 u
Sartorius membrane filter CM approx.	0,75u
Seitz EK pads	1,5 - 1,8 u
Seitz EKS pads	1,2 - 1,5 u
Seitz EKS I pads	1,0 - 1,2 u
Seitz EKS II pads	0,8 - 1,0 u

We were able to confirm that all filtering media impervious to germs, Seitz pads excepted, only keep back small amounts of pyrogens in spite of approximately equal pore diameters. The pyrogen-retaining properties of Seitz pads therfore has nothing to do with a 'straining' effect, and this could not be expected considering the nature of the pyrogens. According to private information received from Prof.O. Westphal, the genuine bacteria-pyrogens and therefore also the test substance used by us, are more or less broken down polysaccharides. (We thank Prof. Westphal for his very informative hints regarding our problems). In their high-molecule components molecule sizes up to 3 1/2 millions were measured and they are therefore comparable to the largest known proteins. According to experience so far gained they should be filterable through hollow space systems such as those examined here. Straining of high-molecule pyrogens by means of ultra-fine filters with correspondingly small pore sizes seems to be feasible. This method is, however, useless in practice since the just as toxic low-molecule pyrogens present cannot be intercepted by that method. The slow rate of filtering of these finely porous filtering media

preparations for intravenous injection.

The conclusion to be drawn is that the effect obtained with Seitz pads can only be attributable to specific powers of adsorption not existing in other filtering agents.

It may be assumed that the active components of the filter pads consisting of cellulose and asbestos is the very finely fiberised asbestos with its large specific surface, the "internal surface" of the asbestos portion of a Seitz EKS II pad of 1000 cm^2 filtering area being about 2000 m^2. In contrast, filters of fritted glass (Schott) or of kieselguhr (Berkefeld) and the membrane filters consisting of a thin frame structure of regenerated cellulose or cellulose esters only have a much smaller inner surface, and this alone could partly explain the quantitative divergencies found. It is also probable that the remarkable efficiency of Seitz sheets is due to special active centres of the asbestos surface.

For a given concentration and given quantity of adsorbent, adsorption will finally lead to a state of equilibrium between adsorbed quantity and the not adsorbed components of the solution. When filtering through a Seitz filter sheet the amount of adsorbent is fixed by the filter area and the asbestos content. Broadly speaking, it will depend upon the concentration of the compounds in the solution when this state of equilibrium is reached in filtering through a given filter area. High concentrations will lead to rapid saturation and therefore a rapid break-through. In weak concentrations larger quantities of liquid will pass through the filtering agent without measurable quantities of the substance to be intercepted by adsorption appearing in the filtrate. Naturally here too, the varying behaviour with respect to adsorption of the substances to be filtered also plays a part. Low-molecule compounds are generally not intercepted as well as the high-mocecular type. Compounds with predominently negative groups are not retained as well as the positive type. Colloids or micells with positive interference potential are well adsorbed by the asbestos which is negatively charged in aqueous media.

For practical pyrogen removal the first conclusion to be drawn from this is: The larger the filter area, the more effective and reliable pyrogen retention will be. In practice, however, the filter size must be limited, in the first place to prevent excessive liquid losses in too large filter sheets, and secondly because the effective substances of the solution existing in low concentrations could be reduced to an undesirable extent by over-dimensioned filters. If the pyrogenic effect of the sheet is known a compromise solution can however easily be found. It should be borne in mind that the differences between concentration of the pyrogens and concentration of the effective substances normally present in drug solutions are very considerable. It was proved that even solutions of high-molecule glucosides in a therapeutically still effective concentration of 0,025% did not undergo practically measurable changes of concentration in filtering through Seitz sheets (H. Wilke p953 (16)).

In comparison, the physiologically effective pyrogen quantities occurring in practical application are approximately of the order of about 0.05 grammes per Cm^3 (corresponding to about 1 million coli-germs per cm^3), i.e. 3 to 4 powers of ten lower.

The main problem, as we see it, was to arrange filtration in such a way that the adsorption capacity of a filtering medium with a definite filter area could be estimated for pyrogens of varying concentrations. It will be appreciated that, considering the extensive test series

carried out, these first had to be confined to a standardised pyrogenic substance. The aqueous solutions of pyrogenic substances of bacterial origin used here can in any case be regarded as model substances for pyrogens liable to occur in manufacture of intravenous preparations.

Since the adsorption tests were, however, only carried out with aqueous solutions, the values found only hold for pure water or for true aqueous solutions of the components. They cannot be applied to colloidal solutions, albumin solutions, etc, probably also not to solutions containing large quantities of compounds with very high molecular weights. We know, for example, from practical experience that serums cannot in all cases be made pyrogen-free by Seitz filtration. It is not yet clear whether this is due to existence of pyrogenic compounds of a different nature or to adsorption displacement by the protein colloids present in considerably higher concentrations.

Freedom of Seitz Sheets form Pyrogens.

Since presence of germs cannot be completely avoided in manufacture of Seitz sheets, it is quite possible that the sheets themselves contain pyrogenic substanes. To find out whether these impurities can be removed in filtering we carried out the following test:

A physiological salt solution prepared with fresh double-distilled water was filtered under sterile conditions through a relatively large filter area of a Seitz EKS sheet. The Seitz porcelain filtering equipment used for the purpose was first made pyrogen-free (without the Filter sheet) by washing with concentrated hydrochloric acid and subsequent rinsing with double-distilled water, and was then sterilised with inserted filter sheet. The first runnings of the filtrate were collected in ampullae cleaned out in the same way and then tested for pyrogens relative to the not filtered solution. The ratio of filtrate quantity to filtering area was 0,8 litres per 1000 sq. cm. If pyrogens are washed out of the sheet at all then these would have led to an enrichment in the first filtrate runnings. In the animal experiment we found:

Original solution: Injection volume 5 cm^3/kg; temperature rise, mean of 3 experiments: 0,3°C.

Filtrate: Injection volume 5 cm^3/kg; temperature rise, mean of 3 experiments: 0.1°C.

This means that the sheets do not surrender pyrogens when filtering normal aqueous solutions.

General Filtering-Test Technique.

All tests described below were carried out with a Seitz sterilising filter, size 6 (according to Manteufel) having a filter area of 20 cm^2. For comparative tests with Berkefeld, Sartorius and Schott filters we chose arrangements with approximately the same filter area. Filtering equipment and ampullae were washed pyrogen-free before sterilisation or filling with solution in double-distilled water with 0.95% salt dissolved in the given quantity. The pyrogen concentrations are given in units per cm^3. The quantity of liquid filtered was each time converted to a filtering area of 1000 cm^2 and entered in the tables as "filtrate per 1000 cm^2". The loading of this area with pyrogens was in each case given in units per 1000 cm^2. The filtrate samples were taken under aseptic conditions and to be quite sure the ampullae were sterilised for 30 minutes at

100°C. and then passed on the pyrogen test. In the tabulated summary of test results the quantities of pyrogen given in the animal experiments were entered in units per kg animal weight. As far as filtrates are concerned, these values are bracketed. Furthermore, the total number of tests for each experiment and the number of positively and negatively reacting animals and the arithmetical mean of maximum temperature rise of the individual experimental animals was also given.

5. Adsorption Filtration of Pyrogens, Partly in Free Existence and Partly Occurring as Constituents of the Destroyed Bacteria.

Pyrogen adsorption as a function of the concentration of the solutions and of the composition of the Seitz sheets.

In the order of filter sheets given in table 3 the asbestos content increases from the very dense Seitz clarifying filtration sheets K 10 to the densest Seitz EKS II sheets. The K 10 sheets are not impervious to germs. For sterilising aqueous drug solutions "EKS" sheets are used. For colloidal solutions, or those containing proteins, the choice is between "EKS I" and "EKS II" sheets (see Wilke (16). These sheets are loaded with solutions of 50 units per Cm^3 (table 3) and 500 units per cm^3 (table 4). These concentrations correspond to a germ content of $5 \cdot 10^7$ and $5 \cdot 10^8$ respectively per cm^3. These high concentrations not occurring in practice were intentionally chosen to determine an upper limit of loadability of the different sheets.

The sterilising sheets loaded with low pyrogen concentrations were found to be practically impervious to germs. The not sterilising sheet K 10, on the other hand, yielded a filtrate with approximately the same febrifacient effect as the original solution. Here the effect of the bacteriabound pyrogens is very noticeable. These cannot make an appearance in filtering through sheets impervious to bacteria. Dependence of pyrogenic activity upon the asbestos concent could not be established with certainty.

As table 4 shows, the sheets are not capable of retaining a 10 time higher pyrogen quantity with an efficiency of practical importance. It is true that a lower reaction temperature is indicated compared to the original solution but in no case was the reduction below 0.6°C.

Table 3:

Loading of different Seitz filter sheets with partly bound pyrogens 50 U/cm^3

Filtering Conditions			
Filtering medium	Pyrogen concentration in U/cm^3	Filtrate volume lit./1000 cm^2	Pyrogen loading U/1000 cm^2
-	50	not filtered	
K 10	50	3	$1{,}5 \cdot 10^5$
EK	50	3	$1{,}5 \cdot 10^5$
EKS	50	3	$1{,}5 \cdot 10^5$
EKS I	50	3	$1{,}5 \cdot 10^5$
EKS II	50	3	$1{,}5 \cdot 10^5$

Animal Experiments

Injected volume	No. of experimental animals	Of these positive reaction	Of these negative reaction	Temperature rise mean	Test result
17	3	2	1	0,6°	+
(50)	3	2	1	0,7°	+
(50)	4	-	4	0,1°	-
(50)	4	-	4	0,2°	-
(50)	4	1	3	0,45°	-
(50)	4	-	4	0,2°	-

Table 4:

Loading of different Seitz filter sheets with partly bound pyrogens 500 U/cm^3

Filtering conditions

Filtering medium	Pyrogen concentration in U/cm^3	Filtrate volume lit./1000cm^2	Pyrogen loading U/1000 cm^2
-	500	not filtered	
EKS	500	3	15.10^5
EKS I	500	3	15.10^5
EKS II	500	3	15.10^5

Animal Experiments

Injected volume	No.of experimental animals	Of these positive reaction	Of these negative reaction	Temperature rise mean	Test result
150	4	4	-	1,4	+
(500)	4	3	1	1,0	+
(500)	4	3	1	0,85	+
(500)	4	4	-	1,0	+

Table 5:

Pyrogen adsorption of Seitz EKS II sheets when loaded with large quantities of liquid.

Filtering conditions

Filter medium	Pyrogen concentration in U/cm^3	Filtrate volume lit./1000cm^2	Pyrogen loading U/1000 cm^2
-	5	not filtered	
EKS II	5	1,5	$0,075.10^5$
"	"	62,5	$3,10 .10^5$
"	"	125	$6,25 .10^5$
"	"	187,5	$9,4 .10^5$
"	"	250	$12,5 .10^5$

Animal Experiments

Injected volume	No.of experimental animals	Of these positive reaction	negative reaction	Temperature rise mean	Test result
14	3	3	-	1,4	+
(14)	3	-	3	0,1	-
(15)	6	-	6	0,1	-
(14)	3	-	3	0	-
(16)	3	-	3	0	-
(14)	3	-	3	0	-

Table 6:

Pyrogen adsorption of Seitz EK sheets when loaded with large quantities of liquid

Filtering Conditions

Filter medium	Pyrogen concentration in U/cm^3	Filtrate volume $lit./1000cm^2$	Pyrogen loading $U/1000\ cm^2$
-	5	not filtered	
EK	5	1,5	$0,075.10^5$
"	"	62,5	$3,10\ .10^5$
"	"	125	$6,25\ .10^5$
"	"	187,5	$9,4\ .10^5$
"	"	250	$12,5\ .10^5$

Animal Experiments

Injected volume	No.of experimental animals	Of these positive reaction	negative reaction	Temperature rise mean	Test result
15	3	3	-	1,1	+
(14)	3	-	3	0	-
(14)	3	-	3	0	-
(14)	3	-	3	0,1	-
(14)	3	-	3	0,1	-
(14)	3	-	3	0,2	-

Table 7:

Behaviour of different filtering media impervious to germs with respect to solutions with partly bound pyrogens.

Filtering Conditions

Filter medium	Pyrogen concentration in U/cm^3	Filtrate volume $lit./1000cm^2$	Pyrogen loading $U/1000\ cm^2$
-	100	not filtered	
Berkefeld W	100	1,5-4,5	max. $4,5.10^5$
Sartorius CM	100	1,5-4,5	max. $4,5.10^5$
Schott G 5	100	1,5-4,5	max. $4,5.10^5$
Seitz EK	100	1,5-4,5	max. $4,5.10^5$
Seitz EKS II	100	1,5-4,5	max. $4,5.10^5$

Animal Experiments

Injected volume	No.of experimental animals	Of these positive reaction	negative reaction	Temperature rise mean	Test result
30	3	2	1	0,7	+
(125)	3	3	-	1,2	+
(125)	3	3	-	1,1	+
(125)	3	3	-	1,4	+
(125)	3	-	3	0	-
(125)	3	-	3	0	-

Table 8: Continuous loading of Seitz EKS sheets with soluble pyrogens 20 U/cm^3

Filtering Conditions

Filter medium	Pyrogen concentration in U/cm^3	Filtrate volume lit./1000cm^2	Pyrogen loading U/1000 cm^2
-	20	not filtered	
EKS	20	1,5-3	$0,3-0,6.10^5$
"	"	24-26	$4,8-5,2.10^5$
"	"	49-51	$9,8-10,2.10^5$
"	"	99-101	$19,8-20,2.10^5$
"	"	149-151	$29,8-30,2.10^5$
"	"	199-201	$39,8-40,2.10^5$
"	"	249-251	$49,8-50,2.10^5$

Animal Experiments

Injected volume	No.of experimental animals	Of these positive reaction	negative reaction	Temperature rise mean	Test result
15	3	3	-	1,0	+
(15)	3	1	2	0,5	+
(15)	3	2	1	0,6	+
(15)	3	1	2	0,5	+
(15)	3	2	1	0,6	+
(15)	3	3	-	0,9	+
(15)	3	2	1	0,8	+
(15)	3	2	1	0,7	+

Table 9:

Continuous loading of Seitz EKS sheets with soluble pyrogens 2 U/cm^3

Filtering Conditions

Filter medium	Pyrogen concentration in U/cm^3	Filtrate volume lit./1000cm^2	Pyrogen loading U/1000 cm^2
-	2	not filtered	
EKS	2	1,5-3	$0,03-0,06.10^5$
"	"	24-26	$0,48-0,52.10^5$
"	"	49-51	$0,98-1,02.10^5$
"	"	99-101	$1,98-2,02.10^5$
"	"	149-151	$2,98-3,02.10^5$
"	"	199-201	$3,98-4,02.10^5$
"	"		

Animal Experiments

Injected volume	No.of experimental animals	Of these positive	negative reaction	Temperature rise mean	Test result
1,5	3	3	-	1,5	+
(1,5)	3	-	3	0,2	-
(1,5)	3	-	3	0,1	-
(1,5)	3	-	3	0,1	-
(1,5)	3	-	3	0,2	-
(1,5)	3	-	3	0,3	-
(1,5)	3	-	3	0,2	-
(1,5)	3	-	3	0,2	-

Table 10: Loading of different filtering media with completely soluble pyrogens.

Filtering Conditions

Filter medium	Pyrogen concentration in U/cm^3	Filtrate volume lit./1000cm^2	Pyrogen loading U/1000 cm^2
-	2	not filtered	
Berkefeld W	2	3,5	$0,7.10^4$
Membranf.	2	3,5	$0,7.10^4$
Seitz EKS	2	3,5	$0,7.10^4$

Animal Experiments

Injected volume	No.of experimental animals	Of these positive	negative reaction	Temperature rise mean	Test result
2	3	3	-	1,5	+
2	3	3	-	2,3	+
2	3	2	1	0,7	+
2	3	-	3	0,2	-

Pyrogen adsorption by Seitz sheets from larger quantities of Liquids

Tables 5 and 6 represent tests in which in one case the densest sterilising sheets "EKS II" and in the other the less active EK-sheet were loaded in a continuous filtering test with a large quantity of liquids containing pyrogens. The liquid quantities were chosen to correspond to the maximum capacity of the filter sheets in sterilising filtration of genuine aqueous solutions. The pyrogen concentration of 5 units was 1 to 2 powers of ten lower than in the preceding test series but with the corresponding germ number of $5 . 10^6$ was still beyond the practically occurring values. At certain intervals during filtration samples were taken for the animal experiments. This provided the means of determining a possible break-through during the filtering period by rising reaction temperatures.

The course of the experiments listed in table 5 shows that even in the final filtrate runnings no temperature rise is indicated at a loading of an EKS II filter sheet of 1000 cm^2 area with 250 lites pyrogen-containing liquid. Even when using in place of the EKS-sheet the EK type which is far less active to germs, no diminishing of activity could be established with certainty, nor was exhaustion of the sheets noticeable, as table 6 shows.

It is interesting to note that in this continuous test the sheets collected the same pyrogen quantity as in the test series table 4. Since in one case completely pyrogen-free filtrates were obtained whereas in the other the filtrates were found to contain large amounts of pyrogens, these tests prove the distinct concentration dependence of adsorption. The total adsorption of pyrogens by the filter sheets is the greater, the lower the pyrogen concentration of the liquid to be filtered in.

Comparison of pyrogen effectiveness of various filtering media impervious to germs.

In the comparative test series the sterilising filter media available in Germany today: Berkefeld cartridges W, Sartorius Membrane Filter, type CM (membrane filter on carrier cardboard), Schott fritted glass G5 and Seitz EK and EKS II sheets were compared with one another in their pyrogenic effect. A continuous test was not carried out. Only a relatively high pyrogen concentration in a small amount of liquid was used for examination. Table 7 shows very clearly the difference between filters containing asbestos with their adsorptive action and those whose sterilising effect is based mainly on 'straining'of the micro-organisms.

No pyrogenic substances were traceable in the Seitz filtrates but considerable amounts in the filtrates obtained with other filtering media. This test again confirms clearly our supposition that pyrogen interception by filters is mainly due to adsorption.

6. Tests With Completely Soluble Pyrogens.

As already mentioned, a large part of the pyrogenic effect is purely attributable to filtering. Only about 10% of the pyrogen-active substances were present in dissolved form. To obtain a clearer picture of the adsorption conditions we carried out analogous test series with true and colloidal pyrogenic solutions. With regard to this, we should like to stress that in the pyrogenic factors of bacterial origin occurring in pharmaceutical practice the pyrogens bound to bacteria bodies are generally not separate from those existing in solution.

Continuous loading of Seitz sheets with dissolved pyrogens.

Our primary aim was to verify test conditions under which in the course of a continuous filtering operation first yielding pyrogen-free filtrates, a gradual break-through occurred. With the two test series represented in tables 8 and 9 carried out with Seitz sheets, we failed to obtain satisfactory results. The concentration was first fixed at 20 units per cm^3 which on the basis of the previous test data should lie in the desired critical zone. Table 8 shows that at the start of filtration distinctly indicated quantities of pyrogens are already present in the filtrate and that with progressing filtration a heavier break-through obviously occurs. Quantitative evaluation of the test results had to be abandoned on account of the non-uniform tendency of the test results.

In the continuous tests with a ten times lower initial concentration, i.e. with 2 units per cm^3, even prolonged filtration did not lead to traceable pyrogens in the filtrate. Here too, a dependence of pyrogen adsorption upon the filter sheet loading could not be proved although we were working in a range in which, according to section 3 of this report, a formula table connection exists between temperature rise in the rabbit experiment and the pyrogen quantity.

Estimation of the pyrogen quantity adsorptively bound in Seitz Sheets

The test results of table 8 and 9 enable us to estimate the proportion of soluble pyrogens bound by the filter sheets. The original solution with 20 units per cm^3 and injected with 15 units per kg animal weight led to a temperature rise of 1.0°C., and a filtrate of this solution applied under indentical conditions in the mean produced a rise of 0.66°C.

This value is still within the fairly regular relation between pyrogen quantity and reaction temperature. It corresponds to a pyrogen content of 0.66 units per cm^3. This would correspond to a reduction of pyrogens due to filtering of 99.5%.

The solution with 2 units per cm^3, which with the same injected volume as above led to a temperature rise of 1.5°C. and is therefore also within the scatter of values, after filtering showed a mean temperature rise of 0.21°C. This corresponds to a pyrogen content so low that it is not determinable with any degree of certainty but on the basis of table 10 can be estimated at 0.045 units and thus indicates a reduction of the original pyrogen content by 98.8%.

Loading of different filtering media with completely soluble pyrogens.

If no pyrogen action could be traced in the test represented in table 7 for straining-type filters, then these filters should allow completely dissolved pyrogens to pass through unhindered. We were only able to prove this by the test series listed in table 10 for the Berkeland filter. A fritted glass filter was unfortunately not available for these tests. On the other hand, the membrane filter which in contrast to the Seitz filter does not supply pyrogen-free filtrates, obviously has only poor intercepting powers.

S u m m a r y.

Without generalising our statements and test results we can say:

The functional connections between pyrogen quantity and temperature rise found in the animal experiment by some writers could only be confirmed with the pyrogenic substances at our disposal for temperature rises up to approximately 0.8-1°C. It is true that higher pyrogen doses caused greater differences of temperature, but with widely scattered values, so that a constant mathematically formulated function could not be established.
The tested pyrogens can be removed from aqueous solutions by adsorption filtration. Of the known filter media impervious to germs only Seitz sheets are effective in this respect. Berkefeld filter candles and fritted Schott glass could not be proved to be effective. Membrane filter efficiency is too low for practical purposes. The action of

Seitz filter sheet is based on adsorption. The quantities of the substances to be removed by filtering can be estimated. About 99% of soluble pyrogens from coligerms occurring in a concentration of 0.5 gamma per cm^3 are intercepted. The adsorbing powers of the sheets with regard to pyrogens depend largely on the concentration. For practical purposes at least 250 litres of a true aqueous solutions can be filtered with 1000 cm^2 filter area if the pyrogen quantity in solution is not greater than 0.1 - 0.5 gamma per cm^3, or if its concentration corresponds approximately to the quantity produced by 100 million coli-germs per cm^3. These pyrogen quantities normally hardly occur in pharmaceutical practice so that it is possible with Seitz filtering equipment to carry out sterilisation and pyrogen elimination of injection preparations simultaneously. These statements are, however, not valid for solutions with a high content in high-molecule or colloidal substances.

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