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# INVESTIGATIONS ON DEPYROGENATION OF DRUGS USING IMMOBILIZED ENZYMES

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## ABSTRACT

A novel technique for depyrogenation of high molecular weight drugs sensitive to heat, acids, alkalies and chemical agents was developed on laboratory scale using sterile immobilized α-amylase. Pyrogenic solution of drug was aseptically treated with immobilized enzyme in the presence of 0.05 % v/v chloroform as a preservative for specific period of time under optimum conditions of temperature, pH and agitation. After a definite period, the drug solution was separated from immobilized enzyme aseptically and heated under vacuum to remove the volatile preservative. Significant decrease in the pyrogenic content of drug solution was observed when tested in rabbits for the presence of pyrogens before and after treatment with sterile immobilized enzyme. The time required for complete depyrogenation was found to be a function of the amount of pyrogens present in the drug. Dry, apyrogenic drug can be recovered from drug solution by suitable means.

## INTRODUCTION

**'Pyrogens'** are substances capable of producing pyrexia. When injected into human body in sufficient amounts, pyrogens will cause a variety of adverse physiological responses - the most common being an increase in body temperature, from which, name 'pyrogen' is derived (Greek **'Pyro'** = fire ; **'gen'** = beginning)[1]. Pyrogens can take on different shapes and sizes depending on the chemical nature of the solution.[2] Chemically pyrogens are **lipopolysaccharides** with molecular weight of the order of 1-2 millions[3]. The presence of pyrogens is most serious in large volume injectables because of the following :

* A large-volume injection will contain a correspondingly large amount of pyrogens ;
* Large volume injections are usually given intravenously, and consequently, the pyrogens will have a more rapid effect ;
* Patients receiving infusion fluids are often dangerously ill and the effect of the rise in temperature could be disastrous.

Therefore, it is of utmost importance to eliminate pyrogens from injections, especially those administered in large volumes intravenously.

The sources of pyrogens in injections may be the solvent, the medicament, the apparatus or the method of storage between preparation and sterilization. Possibly, the most important are **the solvent** and **the medicament.**

Solvent, which in most cases is water, can be made pyrogen free by numerous stills[4] such as **Manesty water still, AMSCO still, Mascarini thermocompression still, Santasolo-Sohlberg Finn-Aqua still** etc. Water can be depyrogenated also by ion-exchange resins, ultra filtration and other methods. **So, production of low cost, high purity, pyrogen free distilled water is not a problem.** Non-aqueous oily solvents can be depyrogenated by heating at 250°C for 30 minutes. Moreover, non-aqueous vehicles are normally used for small volume parenterals.

Many methods available for depyrogenation of drugs are based either on heating or treatment of drugs with acids, alkalies and strong oxidising agents. Drugs can be depyrogenated by dissolving them in

pyrogen free solvent and heating the resulting solution to 250°C for 30 minutes but this method is not applicable to thermolabile drugs. Depyrogenation by treatment with acids, alkalies and strong oxidising agents is not applicable to drugs which are sensitive to these chemical agents.

Adsorptive methods for removal of pyrogens have been demonstrated to remove some level of pyrogens from solutions, but in general, adsorptive methods are not consistent, e.g. activated carbon[2] showed a depyrogenation effect when the carbon was over 5% w/v in concentration and if the pyrogen concentration was below 500ng/ml. However, adsorbent may also adsorb significant quantities of active constituent(s) from the solution. Moreover, pyrogens adsorbed on the absorbent material can unload at any time, thus increasing the concentration of pyrogens in the final solution.

Aqueous solutions exposed to a sterilization dose of 2.5 M rad of radiation from cobalt-60 also did not inactivate the 500 ng/ml level of pyrogens[2].

Another method for depyrogenation of drugs is by ultrafiltration. This method, though developed recently, has gained wide popularity. In this method, the drugs to be depyrogenated are dissolved in a suitable solvent and then subjected to ultrafiltration. This method has got advantage that the pyrogens are physically separated from the drug solution and consequently, degraded products of pyrogens are not left behind in the product as in the case of other methods. However, the use of this method is limited to drugs with low molecular weight. Moreover, ultrafiltration is not a feasible method for large scale processing[5].

It is, therefore, evident that there are still difficulties in removing pyrogens from high molecular weight drugs like **immunoglobulins, interferons, enzymatic casein hydrolysates, albumin preparations, heparin, streptokinase, urokinase, L-asparginase, bacterial hyaluronidase** and **co-carboxylase hydrochloride** etc. Obviously, aforementioned category of drugs can not be depyrogenated by thermal application or by treatment with acids, alkalies and other chemical agents.

In 1947, **Pingert F.P. et al.**[6] patented a process for depyrogenation of high molecular weight drugs sensitive to heat and chemical agents by treatment with amylolytic enzymes. The method comprised of treatment of the drug solution with α-amylase at 43-70°C for a specific period in the presence of suitable volatile preservative such as chloroform, followed by destruction of the enzyme by heating the solution and filtering under apyrogenic conditions. This technique necessiated the use of fresh enzyme every time resulting in high cost and was consequently, industrially unviable. The whole process is represented schematically, in **figure 1.**

In the present studies, the process developed by Pingert F.P. et al. has been reinvestigated by using sterile, immobilized α-amylase so as to permit the repetitive use of same enzyme resulting in steep decline in the overall cost of the process. Number of techniques of immobilization have already been reported in the literature which could facilitate sterile product. In the present process, glutaraldehyde was used as a bifunctional covalent cross linking agent to form sterile insoluble chemical aggregates with α-amylase.

Apart from α-amylase, number of other enzymes such as **ribonuclease**[7], **lysozyme**[8] and **pepsin**[9,10], are also reported to possess the antipyrogenic activity. So, this process offers a vast potential for depyrogenation of wide range of the drugs using immobilized enzymes.

## MATERIALS AND METHODS

### (1) Chemicals and Reagents:

**α-Amylase** and **Bovine serum albumin** were obtained from Sigma Chemical Co. (St.Louis, MO); **Maltose** was procured from DIFCO; **3,5 Dinitrosalicylic acid** was supplied by E. Merck (F.R. Germany) while **Glutaraldehyde (25%)** was obtained from Riedel De Haen AG (Seelze-Hannover). All the other chemicals and reagents used were of analytical grade and obtained from various standard sources.

### (2) A. Preparation of Sterile immobilized α-amylase :

1 gm. of enzyme powder and 1 gm of bovine serum albumin (BSA) were shaken with 20ml of water for injection (to make final

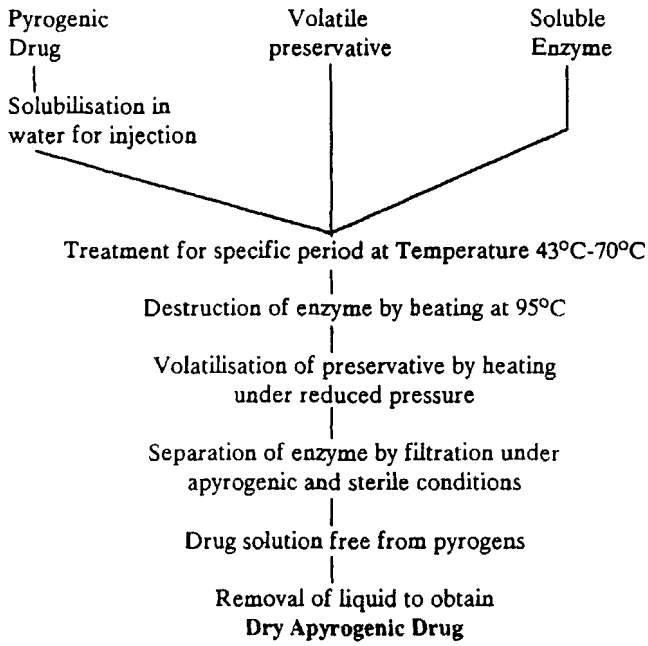


FIGURE - 1

**F.P. PINGERT'S PROCESS FOR DEPYROGENATION OF DRUGS**

concentration 50mg/ml) in a clean, dry, pyrogen free glass tube for 10 minutes. It was centrifuged to get a residue and supernatant. Supernatant was separated and transferred to another clean, dry and pyrogen free glass tube. To this solution, soluble starch was added to make the final concentration of 0.01% w/v. This was kept for one hour.

After one hour, glutaraldehyde was added to make final concentration of 2% v/v. The reaction mixture was left at room tempeature undisturbed for 30 minutes to assist formation of gel. This gel was cut with a blender, homogenized and washed with water for injection long enough to remove free molecules of glutaraldehyde and protein (both were checked in the washings). This homogenized mass was suspended in 20ml of 0.02M sodium phosphate buffer, pH 7.0 and analysed for its activity. The product was tested for its sterility also.

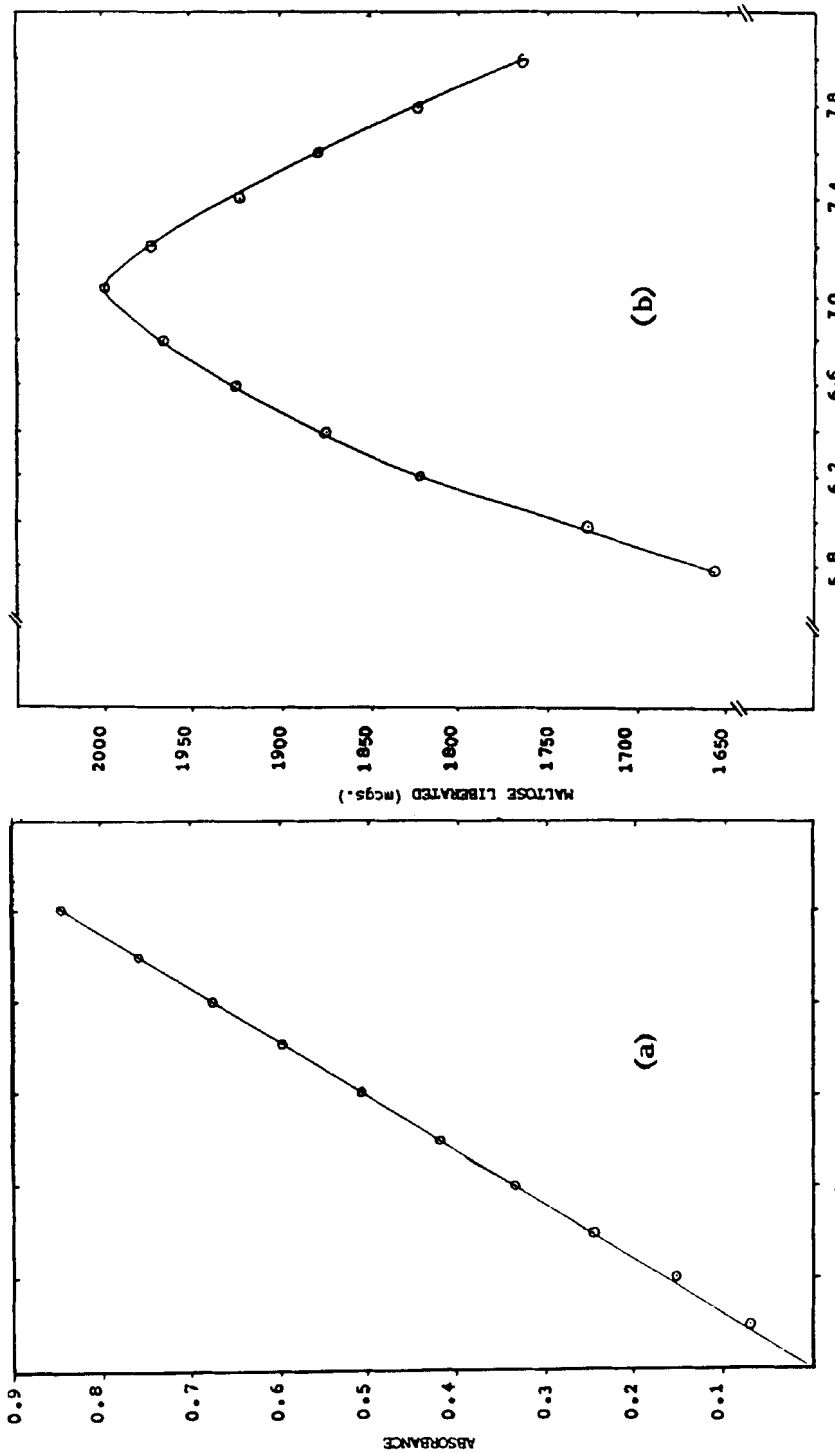


FIGURE 2 (a & b)

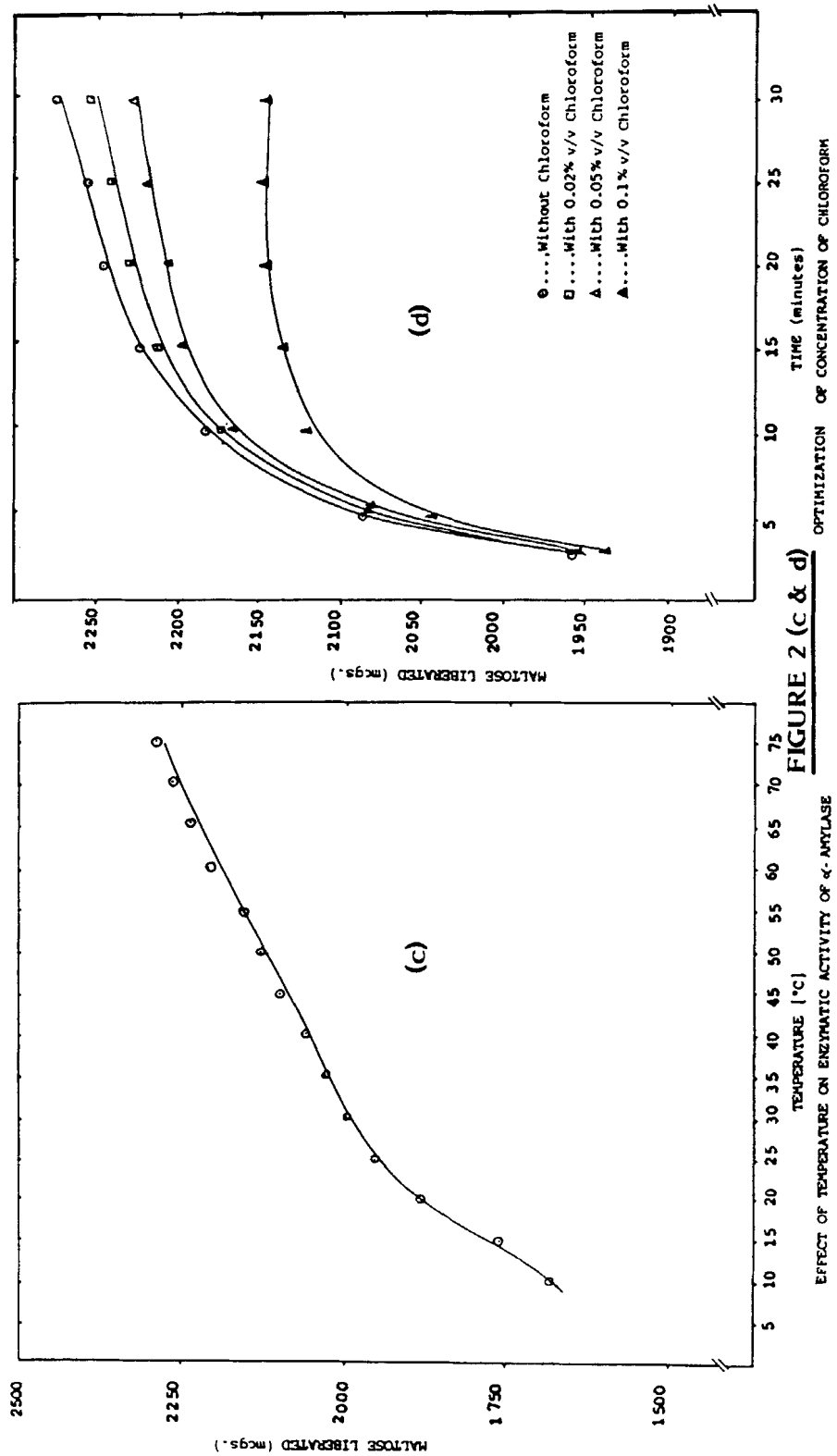


FIGURE 2 (c & d)

**B. Determination of Enzymatic Activity:**

One of the most accurate and commonly used method for measuring activity of α-amylase is that of **Bernfeld**[11] which involves determination of reducing sugars liberated by the enzyme. The maltose liberated from starch is measured by its ability to reduce 3,5 dinitro salicylic acid. The standard curve for maltose determination is shown in **Figure 2a.** A unit of α-amylase activity is that which liberates 1 micromole of maltose per minute at 25°C from 1% soluble starch in 0.02 M sodium phosphate buffer, pH 7.0.

**i) Dinitrosalicylic acid colour reagent:** It was prepared by dissolving 1.0 gm of 3,5 dinitrosalicylic acid in 20ml of 2N sodium hydroxide. 30 gms. of sodium-potassium tartarate was added to it slowly and diluted with glass distilled water to make final volume to 100 ml. It was protected from $CO_2$ and was not used beyond two weeks.

**ii) 1 % Soluble starch solution :** It was prepared by dissolving 1.0 gm soluble starch in 100ml of 0.02 M sodium phosphate buffer, pH 7.0. It was gently boiled to dissolve, cooled and made up the volume to 100ml with distilled water. It was incubated at 25°C for 4-5 minutes prior to assay.

**iii) Enzymatic activity of native α-amylase :** A series of dilutions of enzyme ranging from 20-60 mcgs/ml enzyme powder were prepared. 0.5 ml of various enzyme dilutions was transferred into a series of numbered test tubes. A blank was included with 0.5ml glass distilled water. The tubes were incubated at 25°C for 4-5 minutes to achieve temperature equilibrium. At timed intervals, 0.5ml of 1% soluble starch solution was added at 25°C. These were incubated exactly for 3 minutes and 1ml of DNSA colour reagent was added to each tube at timed intervals. All tubes were incubated in boiling water bath for 5 minutes and cooled to room temperature. 10ml of distilled water was added to each tube and mixed well. Absorbance was measured at 540nm against a blank using **CE 594 CECIL double beam spectrophotometer.** Micromoles of maltose liberated were determined from standard curve of maltose. The activity was expressed as **units/mg** of enzyme powder.

$$\text{Units/mg} = \frac{\text{Micromoles maltose liberated}}{\text{mg. enzyme in reaction mixture x 3 minutes.}}$$

**iv) Enzymatic activity of Immobilized Enzyme :** The activity was determined using the same technique as for the native enzyme. The homogenized mass was suspended uniformly in suitable volume of 0.02 M sodium phosphate buffer, pH 7.0. A definite aliquot (0.5ml) of the suspension was pipetted out (in order to facilitate sampling, the tip of the pipette was cut) and was subjected to assay at 25°C using 1% soluble starch as substrate and DNSA as colouring reagent.

After the development of colour and dilution with water, the solution was subjected to centrifugation to separate the insoluble suspended enzyme. Absorbance was measured at 540 nm against a blank using **CE 594 CECIL double beam spectrophotometer.**

**C. Determination of Activity Yields after Immobilization:**

The activity of α-amylase was determined before and after immobilization using the same procedure as outlined earlier. Activity yields were calculated as the ratio of the activity of an aliquot of insolubilized enzyme to the same aliquot of initial active solution (i.e. solution prior to immobilization). It takes into account both the chemical yield of immobilization and the specific activity of the insolubilized enzyme[12] and is expressed as :

$$\text{Activity yield (\%)} = \frac{\text{Overall activity of the insolubilized enzyme} \times 100}{\text{Overall activity of initial enzyme solution}}$$

This process was repeated twice and the results are compiled in **Table 1.**

**D. Sterility Testing :**

In order to determine whether the aforementioned immobilization technique yielded sterile immobilized enzyme or not, immobilized enzyme was subjected to sterility testing for bacteria and fungi. The media used for these were Nutrient Broth and Fluid Sabourand media for bacteria and fungi respectively. The tubes were incubated at 25°C for fungi and at 37°C for bacteria for a period of 14 days. The results are recorded in **Table-2.**

**E. Depyrogenation activity of Immobilized α-Amylase and Testing for reproducibility :**

In order to simplify and quantify the results, it was necessary that the source should contain a definite and preferably known amount

TABLE 1

ACTIVITY YIELDS AFTER IMMOBILIZATION OF α-AMYLASE

| Parameter | Set Number | | |
|---|---|---|---|
| | I | II | III |
| pH of immobilization | 7.0 | 7.0 | 7.0 |
| Activity before immobilization | 16.3 | 16.3 | 16.3 |
| Activity after immobilization | 6.20 | 6.52 | 6.34 |
| Percentage of remaining activity | 38.04 | 40.0 | 38.9 |
| Mean Activity Yield (%) | | 38.98 | |

TABLE 2

STERILITY TESTING OF IMMOBILIZED ENZYME

| S.No. | Test tube number | Growth observed after 14 days | |
|---|---|---|---|
| | | Nutrient Broth media | Fluid Sabourand media |
| 1. | Positive control | Positive | Positive |
| 2. | Negative control | Negative | Negative |
| 3. | One | Negative | Negative |
| 4. | Two | Negative | Negative |
| 5. | Three | Negative | Negative |
| 6. | Four | Negative | Negative |
| 7. | Five | Negative | Negative |
| 8. | Six | Negative | Negative |

of pyrogens. Since ∝ -amylase, an amylolytic enzyme is active against pyrogens as well as soluble starch, therefore, the activity of ∝-amylase against soluble starch can be considered as an indirect measure of its antipyrogenic activity. Soluble starch was selected for determination of enzymatic activity of native as well as immobilized enzyme. Bacterial vaccines from gram-negative bacteria are highly pyrogenic and since the number of microorganisms per unit volume are standardised, therefore, these vaccines can act as an excellent substrate for determination of antipyrogenic activity.

Typhoid - Paratyphoid Vaccine (T.A. Vaccine) was used as a source of pyrogens for final confirmation of antipyrogenic activity of immobilized enzyme. Each ml of T.A. Vaccine **(CRI, Kasauli)** used contained **Salmonella typhi - 1,000 million** and **S. paratyphi A - 500 million.**

Conventional rabbit method as described in Indian Pharmacopoeia, 1985 was used to detect the presence of pyrogens in the sample. The rise in body temperature of rabbits after injection of sample was taken as comparative criteria i.e. greater rise in temperature was taken as indicative of higher amounts of pyrogens and vice versa.

**i) Preparation of Pyrogenic Sample:** A single, 30ml vial of Typhoid-Paratyphoid vaccine was sterilised by autoclaving at 121°C for 30 minutes. Since autovlaving under these conditions is known to destroy only bacteria and not pyrogens, the same vial was used as a source of pyrogens. For preparation of the pyrogenic sample, 1.0ml of contents of vial were mixed with 99ml of pyrogen free normal saline, pH 7.0, under aseptic conditions.

**ii) Treatment of Pyrogenic Sample with Immobilized Enzyme:** 100ml of above sample was aseptically treated with approximately 1.0gm of immobilized enzyme in a well closed, sterile and pyrogen-free container, capable of retaining sterility, for a period of 12 hours at 25°C in the presence of chloroform (0.05% v/v) as a preservative.

**iii) Separation of Immobilized Enzyme:** The immobilized enzyme was separated from the reaction mixture by means of filtration under aseptic, pyrogen-free conditions using pyrogen-free Whatman filter paper **(pre-treated with hydrogen-peroxide to remove pyrogens).** The separated enzyme was stored in apyrogenic sodium phosphate buffer,

TABLE 3

DEPYROGENATION ACTIVITY OF IMMOBILIZED ENZYME

| S.No. | Description of Sample | Maximum Temperature rise observed in three rabbits (°C) (1) | (2) | (3) | Summed Response (°C) |
|---|---|---|---|---|---|
| 1. | Blank | 2.0 | 2.2 | 1.9 | 6.1 |
| 2. | S-1 | 0.3 | 0.4 | 0.2 | 0.9 |
| 3. | S-2 | 0.5 | 0.1 | 0.3 | 0.9 |
| 4. | S-3 | 0.2 | 0.2 | 0.3 | 0.7 |

pH 7.0, in the presence of chloroform in a concentration of 0.05% v/v as a preservative. The separated solution was heated to remove preservative, autoclaved and tested for its pyrogenic activity in rabbits. The test was carried out as per specifications of **I.P.'85.** The rabbits were injected with a dose of 5ml per kg body weight.

The separated immobilized enzyme used for earlier experimentation was retreated with another pyrogenic sample of normal saline under similar conditions as described previously. This was repeated twice and the treated samples were checked for their pyrogenic activity in rabbits. The following samples were tested for their pyrogenic activity in rabbits as per **I.P.'85** specifications:

**Sample no. 1 :** Blank containing 1.0ml of vaccine contents in 100ml of normal saline.

**Samples no. 2,3 and 4 (S-1, S-2 and S-3):** Pyrogenic samples treated with immobilized enzyme as per modified process and tested for pyrogens The samples were treated with immobilized enzyme for a period of 12 hours. This was repeated twice. The results are reported in **Table 3.**

## RESULTS AND DISCUSSION

α-Amylase, as mentioned earlier, has got the depyrogenation activity. The native enzyme has got optimum pH approximately at 7.0 Enzymatic activity increased with increase in the temperature up to 70°C in case of α-amylase from bacterial origin. The immobilized enzyme offers three distinct advantages[13] over the soluble enzyme: Reusability, Greater stability and Decreased susceptibility to interfering substances.

In the present process of immobilization, glutaraldehyde was used as a bifunctional covalent cross linking agent to form insoluble aggregates of α-amylase. In this process, bovine serum albumin was chosen as an auxillary soluble protein to the enzyme solution because of its lysyl residue content. Soluble starch was used as a protective thus preventing the active sites of the enzyme to come in the direct contact with glutaraldehyde.

Usually there is some loss of activity of enzyme once it is immobilized. With the aforementioned process of immobilization of α-amylase, **activity yields of the order of 38-40% were obtained (Table 1).**

The process of immobilization was also tested for its efficacy to yield a sterile product. The chemical aggregates of α-amylase obtained after cross linking with glutaraldehyde were tested for the presence of living microorganisms (both bacteria and fungi) in the presence of their respective growth supporting media i.e. Nutrient broth for bacteria and Fluid sabourand media for fungi. It was found that the **process could yield sterile product** as evident from the results compiled in **Table 2.**

After immobilization of enzyme, the various parameters affecting the activity of α-amylase like pH, temperature, presence of preservative etc. were also studied using soluble starch as substrate to get optimum conditions for depyrogenation. Based on the results of activity yields after immobilization, a suspension of immobilized enzyme possessing about 60 units/ml activity was prepared in water for injection and used for the optimization of various experimental conditions.

**Influence of pH :** The enzyme suspensions were prepared at different pH ranging from 5.8 to 8.0. Likewise, 1% soluble starch solutions, taken as substrate, were prepared in the above mentioned pH range. The activity of immobilized enzyme was determined at different pH taking every reading in triplicate. It was found that the **optimum pH remained approximately at 7.0.** The results are illustrated in **Fig. 2b.**

**Effect of temperature :** The activity of immobilized enzyme was determined at different temperatures ranging from 10°C to 75°C. It was found that the immobilized enzyme was fairly stable in wide temperature range **(Figure 2c).** Since the present process deals with thermolabile drugs, therefore, the ideal working range in which the enzyme also possesses fairly good activity was selected as **20-40°C.**

**Influence of preservative :** Although exceptionally high antimicrobial activity of glutaraldehyde yielded sterile immobilized enzyme but the need of a suitable preservative to retain the sterility could not be ignored. The preservative should be such that it does not interfere with the enzymatic activity of α-amylase and its removal from the final, treated product is easy. Taking these factors into consideration, chloroform was chosen as preservative. The effect of the various concentrations of chloroform (from 0.02% to 0.1% v/v) was investigated as a function of time on the activity of immobilized enzyme taking every reading in triplicate. It was found that, **chloroform in a concentration of 0.05% v/v has insignificant inhibitory effect on the activity of α-amylase and maintains sterility effectively.** The results are illustrated in **Figure 2d.**

Presence of pyrogens in parenterals is not desirable. But their removal from a drug is also not easy owing to some of their typical properties like -

i) **Thermostability :** to destroy some types, temperatures much higher than those used in the official sterilization processes are necessary.

ii) **Water-solubility :** therefore, they are not removed by the usual types of bacteria-proof filters.

iii) **Unaffected** by the common bactericides.

iv) **Non-volatility :** which facilitate their separation from water

by distillation. 'Distillation still' designs are suitably modified and are provided with entrainment separators which do not allow water droplets to go along with steam and thus providing apyrogenic distillate.

But the same cannot be applied to drugs. In fact, on the category of drugs of interest (i.e. high molecular weight drugs sensitive to heat and chemical agents), no conventional method like heat, acid/alkali treatment, treatment with strong oxidising agent or filtration is applicable in order to render them free from pyrogens. For such a category of drugs, the present process may provide useful way to get rid of pyrogens. The process is illustrated schematically in **Figure 3.**

**The process consists of the following steps :**

* Immobilizing the enzyme by suitable technique capable of yielding sterile product.
* Bringing sterile pyrogenic drug solution in contact with immobilized enzyme in the presence of a suitable volatile preservative in an appropriate concentration.
* Incubating under optimum conditions of temperature and pH for specific period.
* Separation of drug solution from immobilized enzyme by means of filtration under sterile, apyrogenic conditions.
* Heating the drug solution under reduced pressure to volatilise preservative.
* Removal of solvent from drug solution by suitable means to obtain dry apyrogenic drug.

**One of the major drawbacks of Pingert's process was its high cost.** In that process, α-amylase used for depyrogenation could be used only once and then it had to be destroyed by heat coagulation in order to separate it from drug solution. But in the present process, the α-amylase was immobilized by chemical aggregation using glutaraldehyde and thus, the same enzyme can be reused till it retains its activity. However, it has to be periodically checked for the retained activity and is to be supplemented with afresh immobilized enzyme or the time period for reaction can be increased to compensate the loss of activity on storage. But all these parameters have to be optimized before adopting it commercially.

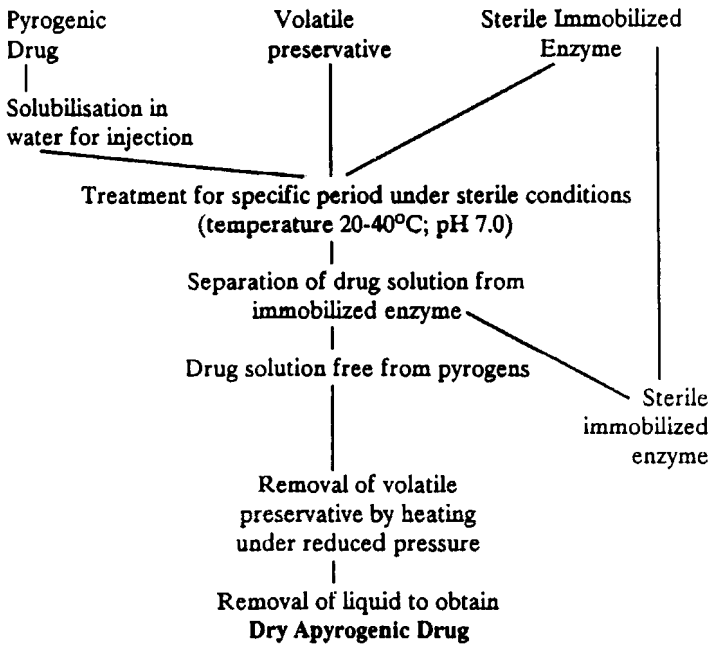


FIGURE - 3

**PROPOSED TECHNIQUE FOR DEPYROGENATION OF DRUGS**

The depyrogenation capability of immobilized enzyme was checked by using previously autoclaved **Typhoid-Paratyphoid (TA) Vaccine** - a vaccine from gram-negative bacteria. This was selected as a source of pyrogens because vaccines are standardised preparations and known to contain a definite amount of microorganisms. To further rule out the possibilities of any variation on this aspect, the contents of the same vial were used. 1 ml of vial contents was mixed with 99ml of sterilized normal saline and this mixture was taken as a pyrogenic sample. This was treated with sterile immobilized enzyme for a period of 12 hours with constant agitation. After completion of this period, the sample was treated as per the process. Both treated and untreated samples were tested in rabbits for pyrogenicity. The same enzyme was also tested for the reproducibility i.e. it was treated repeatedly with pyrogenic normal saline, samples were processed and tested in

rabbits. The observations are compiled in **Table 3.** The hyperthermia produced after injection was taken as criteria for pyrogenicity. Greater rise in body temperature was taken as indicative of higher pyrogenic level in the sample tested and vice versa. From these observations, it is quite clear that the **pyrogenic content has been substantially reduced after treatment with the immobilized enzyme.**

## CONCLUSIONS

Depyrogenation of high molecular weight drugs sensitive to heat, acids, alkalies and chemical agents was discovered as early as in 1947 by Pingert F.P. et at. Though this method had lot of potential, it could not be adopted commercially because the process involved destruction of enzyme resulting in steep increase in the cost.

In order to lower the cost, the above mentioned process was investigated after immobilization of enzyme thus allowing the repetitive use of the same enzyme. Immobilization of α-amylase was carried out by chemical aggregation using glutaraldehyde. The product obtained was also sterile owing to the exceptionally high antimicrobial activity of glutaraldehyde. Sterility of the product prevented further formation of pyrogens. Activity yields of the order of 38-40% were obtained.

The various factors affecting the enzymatic activity of immobilized enzyme were studied. It was found that the immobilized enzyme possess optimum activity at pH 7.0, in the presence of 0.05% v/v chloroform as a preservative in the temperature range of 20-40°C. There was complete depyrogenation of pyrogenic sample of normal saline when brought in contact with immobilized enzyme for twelve hours, under these conditions. In fact, the time required for complete depyrogenation was found to be a function of the amount of pyrogens present in the sample.

This process has got distinct advantage over the Pingert's process because of non-destruction of enzyme, there will be no necessity to use fresh enzyme as a raw material for every batch resulting in steep decline in the overall cost of the process. Steep reduction in the cost is likely to render this process commercially competitive. Apart from α-amylase,

other enzymes such as **ribonuclease, lysozyme** and **pepsin** too, have been reported to possess depyrogenating activity.

Though the process has been optimized using pyrogenic sample of normal saline at laboratory scale, the work using the actual high molecular weight drug sensitive to heat and chemical agents is still in progress.

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## REFERENCES

1. Akers, M.J., "Parenteral Quality Control: Sterility, Pyrogen, Particulate and Package Integrity testing", Marcel Dekker, Inc., (1985), p.79.
2. Meltzer, T.O., "Filtration in Pharmaceutical Industry", Marcel Dekker, Inc., (1987), p.919.
3. Elizabeth, in "Pyrogens and Fever - A Ciba Foundation Symposium", Wolstenholme, G.E.W. and Birch, J., eds., Churchill Livingstone, Edinburgh and London (1971), p.33.
4. Myers A.J. and Hanblenton R., in "T.B. of Hospital Pharmacy", Allowood M.C. and Fell J.T., eds., Blackwell Scientific Publications, London, (1980), p.144.
5. Avis, K.A., in "Remington's Pharmaceutical Sciences", Arthur Osol edi, Mack Publishing Co., Easton, 17th ed., (1985), p.1523.
6. Pingert F.P. et al. : U.S. Patent 2,432,970 (1947).
7. Sadao, O: Japanese Patent 7,622,887 (1976).
8. Caselli, P. and Callierio, C., Nature, 188, 238 (1960).
9. Plisser, M: French Patent 1,483,901 (1967).
10. Zimmermann, G.: German Patent DD 157,417 (1982).
11. Bernfeld, P., in "Advances in Enzymology", Vol.II, Nord. F.F. edi., Interscience Publications, New York (1951), p.379.

12. Broun, G.B. in "Methods in Enzymology", Vol. 44, Mosbach,K. edi., Academic Press, New York (1976), p.267.

13. Guilbault, G.G., "Handbook of Enzymatic Methods of Analysis, Marcel Dekker, Inc. (1976), p.446.