CHAPTER FOUR. Fluorescence in gelatine.

INTRODUCTION.

The only mention of gelatine and fluorescence in the literature appeared to be the Gelatine and Glue Research Association Research Report A33 (Thornton, 1966) in which home-made equipment using filters was used to demonstrate gelatine sol and gel fluorescence depolarisation.

More recently there had been many reports on collagen fluorescence. These included:

Tanaka, Avigad, Eikenberry and Brodsky (1988) reported an increase in fluorescence with time, for rat tail collagen incubated in the presence of ribose at 35°C for up to two weeks. They showed that a similar increase in collagen fluorescence occurred as a result of animal age. In addition, when soluble collagen was incubated with ribose, dimers and trimers of the collagen α-chain were formed which confirmed that the fluorescence was associated with cross-linking.

Sell and Monnier (1989) isolated the "pentosidine" collagen cross-link from human collagen (dura mater), correlated the increase in quantity with age and elucidated the structure of the cross-link by synthesis which involved heating equimolar quantities of lysine, arginine and ribose at pH 7.2 and 80°C for one hour. They claimed that similar treatment of a mixture of lysine, arginine and glucose had not resulted in the formation of a fluorescent product hence there had been a "problem" in accounting for the source of the ribose used in cross-link formation.

Monnier, Sell, Miyata and Nagaraj (1990) used the 335/385 nm fluorescent collagen "pentosidine" cross-link as the basis of a theory that senescence was the result of the Maillard reaction between functional proteins and ribose, the resulting impaired functionality of the protein being seen as the well known manifestations of age.

Dyer, Blackledge, Katz, Hull, Adkisson, Thorpe, Lyons and Baynes (1991), in

a review "The Maillard reaction in vivo", stated that "since pentosidine contains only 5 carbons from the sugar component, its formation from glucose must involve the loss of a carbon atom either from glucose itself or from a later intermediate in the reaction. ...while its (pentosidine's) origins from ribose v/s glucose and other sugars may be uncertain....." indicated that there had been evidence for the formation of pentosidine from glucose attributed to Baynes *et al.* (1990).

In the study of Uchiyama, Ohishi, Takahashi, Kushida, Inoue, Jugie and Horiuchi (1991) human articular cartilage was treated with collagenase. The fluorescence of the digests was shown to correlate with age. Furthermore, the authors demonstrated that the 295/395 nm fluorescence was due to the pyridinoline collagen cross-link and that the amount was largely invariant with age. However, the 335/385 nm fluorophore was shown to be identical with the "pentosidine" of Sell and Monnier (1989) and the amount increased linearly with age.

Eyre (1987) gave the details of the methodology for the isolation of hydroxylysylpyridinoline (HP) and lysylpyridinolin (LP) cross-links of collagen which were fluorescent. It was stated that these cross-links could be detected in PAGE gels using an excitation wavelength of 330 nm and an emission wavelength of 395 nm. However, these cross-links were not found in skin collagen (Yamauchi Woodley and Mechanic, 1988), hence, they should not interfere with the detection of the pentosidine cross-link in hide gelatine using fluorescence.

Based on the observation that gelatine colour also increased with animal age (*loc sit*) it appeared reasonable to hypothesize that gelatine colour could be the result of the Maillard reaction (*in vivo*). One method of verifying this would be to observe the fluorescence of gelatine. If gelatine exhibited fluorescence at 335/385 nm and the fluorescence increased with colour and animal age, it would follow from the work of Uchiyama *et al.* (1991) that the colour could be attributable to the formation of pentosidine collagen cross-links. If this was indeed the case then, as with collagen, gelatine should darken and fluorescence should increase on incubation with glucose or ribose sugars.

Although a number of texts (Munck and de Francisco, 1989; Lacowits, 1983) on fluorimetry were studied, "Fluorescence Analysis in Foods" (Munck and de

Francisco, 1989) appeared to be the most relevant with respect to the advantages and disadvantages of the method:

1. The main advantages were the extreme sensitivity and excellent specificity of the method. Only a few of the compounds absorbing light emit fluorescent light, and in contrast to spectrophotometry, two wavelengths were used in fluorimetry (Munck and de Francisco, 1989a).
2. The main disadvantage (Munck and de Francisco, 1989b) of fluorimetry was the phenomenon of "quenching" or reduction of the emission intensity. Quenching could be caused by high concentration of a fluorophore. This causes absorption of the emitted fluorescence and attenuation of the excitation beam in the areas of the solution in front of the detection system. Quenching could also be caused by oxygen, impurities and temperature and pH. In the current study, temperature in particular could have been a problem as there were no facilities to control sample temperature (temperature increase causes fluorescence decrease). Sell and Monnier (1989) also noted that the fluorescence of pentosidine was pH dependent and that the fluorescence was completely quenched at pH 9.
3. Another disadvantage (Munck and de Francisco, 1989c) of fluorimetry as an analytical tool was its serious dependence on environmental factors such as temperature, pH, ionic strength, and previous exposure to photochemical decomposition (sun light, fluorescent laboratory lights etc.). The latter required that fluorescence be measured immediately after excitation and accounted for the fact that the intensity of gelatine fluorescence was observed to decrease with time of exposure (i.e. a steady reading of fluorescence intensity was not obtained and the readings that were recorded were instantaneous readings taken immediately after inserting samples into the measuring compartment of the instrument).

Hence, whereas fluorimetry was advantageous from the point of view of product identification it was evident from the above that the probable error of quantitative results was large unless all variables were rigorously controlled.

From the report by Odetti, Pronzato, Noberasco, Cosso, Traverso, Cottalasso and Marinari (1994), it appeared that fluorimetry was an accepted technique for studying pentosidine's relationship to ageing phenomena.

METHODS AND MATERIALS.

Unless otherwise stated reagent grade chemicals were used throughout this part of the study.

Choice of solution concentration.

Dyer *et al.* (1991) pointed out that for every mole of pentosidine there must be an additional 1000 cross-links in the dimerised protein (ß chains). Hence, one must expect the concentration of pentosidine to be low, and a high concentration of gelatine would be required. High concentrations would however be expected to give practical problems associated with gelation hence a compromise, using a nominal concentration of 1% w/v, was decided upon (i.e. 0.20 g dissolved in 20 ml distilled water). This concentration was found to be satisfactory as it gave fluorescence intensities of about 50 for calf skin gelatine to about 200 for old animal skin gelatine. The high fluorescence intensities encountered with gelatines after incubation with ribose or glucose required further dilution to 0.01% concentration (0.1 ml 1% w/v solution added to 10 ml distilled water).

Instrumentation.

The instrument used in this study was a SCHIMADZU RF-5000 Spectrofluorophotometer (ILSA (Pty) Ltd. PO Box 8166, Johannesburg.) capable of scanning both emission and excitation spectra. In "Multispectra" mode the instrument was preprogrammed to run 5 scans of the emission spectrum at 5 preset intervals of excitation wave length. The scans were then presented together on one printout. This facilitated the rough determination of the existence of excitation and emission peaks. These peaks were refined by running scans of the excitation and emission spectra at very slightly different wave lengths. For readings at fixed wavelengths the excitation and emission wavelengths could be set and the fluorescence intensity read on the computer screen (CRT). No facility to download data for storage or reanalysis was available. The only method of data storage was a printout of a scan using the attached thermal printer or the manual recording of numerical data. All the determinations were carried out using 5 nm excitation and emission slit widths.

Fluorescence determination.

Aliquots (0.20 g) of gelatine powder were weighed into 50 ml clear glass bottles. Distilled water (20 ml) was added by pipette. Screw caps were applied to the bottles and the gelatine was allowed to swell for 20 min. The bottles were then placed in a 40°C waterbath and swirled several times over 30 min. to achieve complete solution of the gelatine. For the measurement of the sample fluorescence intensity on the spectrofluorophotometer, a 1 cm quartz cuvette was rinsed three times with the sample and then filled and placed in the instrument's cuvette holder. The sample compartment was closed and then the sample was scanned, or the fixed wavelength intensities were read from the CRT.

The effect of incubating gelatine with glucose.

1. Outline.

Two pale, unblended, first extraction, Type B commercial gelatines were incubated with glucose at 50°C and at pHs 6 and 9 for up to 12 weeks. The changes in colour and fluorescence were monitored with time. The collagen/glucose Maillard reaction was known to be very slow presumably due to the low concentration of glucose in the aldohexose configuration (0.002%). (Tanaka *et al.* 1988). The temperature of 50°C was chosen both to accelerate the reaction and to reduce markedly the probability of interference by microbiological proliferation. Tanaka *et al.* (1988) chose to add thymol as a bacteriastat. The pH of 6 was chosen as this was close to the physiological pH (Lawrie, 1985) and the pH of 9 was chosen as this was the pH used for the "Formol" titration determination of free amino groups in protein (Cobbet Gibbs and Leach, 1963 and Leach, 1965). At this pH the amine/aldehyde reaction was virtually instantaneous due to the absence of a charge on the ϵ amino side chain of lysine.

In the planning of this experiment the development of very high intensity fluorescence was not anticipated hence determination of the fluorescence on 0.01% gelatine solutions was an added procedure as was the determination of the pH of the reaction mixture at the same time that the fluorescence was determined.

2. Controls.

The following controls were also planned:

a) Glucose at pH 6 and 8.75 was incubated at 50°C to ensure that "glucose caramelization" could not be a factor in any colour change.

b) Each time an incubated sample was taken for assessment a fresh sample of the gelatine was dissolved to act as a control on instrument performance.

c) Gelatine at pH 6 and 8.75 were included as the effect of 50°C incubation on the colour of gelatine solutions had not been documented.

3. Experimental detail.

Two similar commercial Type B bovine hide gelatines (ex Davis Gelatine Industries (Pty) Ltd, P.O. Box 5019, West Krugersdorp, 1742.) which had been extracted in 1992 and 1994, were used. Gelatines 155/1 and 877/1, with similar Bloom gel strengths and colours, were chosen. Aliquots of gelatine (25 g) were mixed with 9 g glucose (BDH Laboratory Reagent Grade) and 150 ml distilled water in a 500 ml beaker. The gelatine was allowed to swell for 30 min. The mixture was then placed in a 50°C water-bath and stirred occasionally over 30 min. until the gelatine had dissolved and the solution was homogenous. Using a pH meter, 0.28N NaOH was added from a burette with stirring until the required pH was reached - 6.0 or 8.75. The pH of 8.75 was used because the quantity of NaOH consumed was greater than anticipated. The contents of the beaker were then adjusted with distilled water to a net weight of 250 g. After mixing well at 50°C, 15 ml aliquots were dispensed into sterile Mc Cartney bottles which were then placed in an oven thermostatically controlled at 50°C ± 2°C.

25 g	gelatine.	10.0%
9 g	glucose.	3.6% or 0.2 Molar
216 g	NaOH soln. + distilled water.	86.4%
250 g		

On commencement of the experiment and at weekly intervals, one sample of each

of the 8 lots (gelatins 155 and 877 at pHs 6.0 and 8.75 both with and without glucose) was taken. From each sample aliquots of 7.15 ml and 2.0 ml were pipetted into sample tubes for colour and fluorescence determinations. Enzyme solution (ES1) was made up by taking 0.52 ml Alcalase 0.6L (Novo Enzymes. Enzymes S.A. (Pty) Ltd. PO Box 651216. Benmore 2010) and diluting it to 200 ml with distilled water in a volumetric flask.

The first tube containing 7.15 ml gelatine solution was diluted by the addition of 3.85 ml freshly prepared ES1 solution containing 0.01 ml Alcalase. The mixture was held at 40°C for 1 hr to complete the hydrolysis reaction. After filtration of the sample through a 0.45 micron membrane filter and using ES1 as the blank, the absorbance was scanned between 400 nm and 700 nm. The area under the absorbance curve after moving the base line to the 700 nm absorbance, was taken as the measure of the gelatine colour.

Note: Gelatine reaction mixture (7.15 ml) contained 0.715 g gelatine and 6.15 ml water. The addition of 3.85 ml ES1 soln. gave a water volume of 10.0 ml. .715/10.715x100 = 6.67% gelatine solution, the chosen concentration for colour determinations.

A control consisting of 0.714 g gelatine, 3.85 ml ES1, and 6.15 ml water, was measured each day to insure that the instrument and procedure were behaving normally. This sample was allowed to swell for 30 min. and dissolved at 40°C. A 1 hr reaction time at 40°C was allowed before the sample absorbance was determined.

The second tube containing 2.0 ml of reaction mixture was diluted with 18.3 ml distilled water for fluorescence determination as detailed above. When the fluorescence intensities increased to >1000 units the instrument ceased to respond and it was decided to dilute the sample to 0.01% by taking 0.1 ml of the above solution and adding this to 10 ml of distilled water. Later the apparent fluorescence of the 1% samples decreased due to concentration quenching as shown by the continued increase in 0.01% solution fluorescence.

Note: Incubated gelatine reaction mixture (2 ml) contained 0.2 g gelatine and 1.7 ml of water which had to be increased to 20 ml for the fluorescence determination at 1% concentration.

Tanaka *et al.* (1988) noted 370/445 nm fluorescence in ribose treated collagen.

hence this pair of wave lengths was also monitored with the glucose treated gelatine.

THE EFFECT OF INCUBATING GELATINE WITH RIBOSE.

1. Preliminary study No 1.

Based on the researches of Tanaka *et al.* (1988) the effects on the absorbance spectrum of gelatine, caused by incubating gelatine solution at pH 5 and 9 with glucose and ribose, were determined.

Two 6.67% gelatine solutions were made up using "Colour Standard" gelatine (Colour 8, Clarity 10) at 45°C. One of these was adjusted from pH 5.0 to 9.0 using approx. 10 ml 0.2N NaOH solution. Three test tubes were filled with 10 ml aliquots of the pH 5 solution and three tubes were filled with 10 ml aliquots of the pH 9 solution. One of the pH 5 and one of the pH 9 tubes was treated with a small amount of dry glucose after which the tubes were inverted several times to achieve solution and mixing. Similarly two tubes were treated with ribose and the two tubes that received no addition acted as controls. The tubes were labelled, capped with aluminium caps and then placed in a 44°C oven. After 7 days and after 14 days 2 ml aliquots were withdrawn from each tube for measurement of absorbance using the Jenway Colorimeter (Jenway Ltd. Dunmow, Essex, UK) and Filter No 2 with water as the blank. These samples were also used to determine the absorption spectrum when required.

2. Preliminary study No 2.

The object of this series of experiments was to observe the effects of the gelatine-ribose interaction at 37°C on colour and other gelatine quality parameters at pH ±5 and pH ±8. The following solutions were made up in quadruplicate in Bloom bottles:

Solution 1 (pH ± 5)

Gelatine 23/2L	7.50g	
Ribose	3.15g	(Tanaka *et al.*, 1988, 0.2M)
Thymol	0.10g*	(arbitrary quantity of preservative)
Distilled water	105.0g	

Solution 2 (pH ± 8)

Gelatine 23/2	7.50g
Ribose	3.15g
Thymol	0.10g*
0.2N NaOH	15 ml (by pipette = 15.09 ± 0.01g)
Distilled water	90.0g (NaOH + water = 105.0g)

* was not immediately soluble at 40°C, but globules disappeared with time at 37°C.

The samples were dissolved at 40°C and then incubated at 37°C. After the required period of incubation, samples were taken and placed in the 10°C Bloom bath for 16 to 18 hr and then the Bloom strengths were measured. The samples were then melted and the other normal quality measurements were made on the solution as well as the absorbance of the 6.67% solution using the Jenway Colorimeter (*loc cit*) with distilled water as the blank.

3. Detailed study.

In this study ribose and gelatine were incubated at 37°C and at pHs 6 and 9 for up to 12 days. Due to the higher reactivity of ribose as found in the preliminary study No 1, incubation was carried out at 37°C and samples were removed from the oven daily for measurement of colour and fluorescence. Due to the lower molecular weight of ribose the following mixtures were made up using gelatines 155/1 (RA @ pH 6, RB @ pH 9) and 877/1 (RC @ pH 6, RD @ pH 9):

Gelatine	25g	
Ribose	7.5g	
Thymol	0.25g	(0.1% as preservative)
Water	150g	
0.28N NaOH	qs	(± 17 ml to pH 6 or ± 45 ml to pH 9)
Water	qs	to 250g total weight.

Controls samples (gelatine 155/1, RE and RF and gelatine 877/1, RG and RH) in which ribose was replaced by water, were made up similarly in order to be able to assess the effects of the experimental conditions on colour and fluorescence.

Pentosidine synthesis.

Solutions of L-lysine hydrochloride and L-arginine hydrochloride (ex Sigma Chemical Corp.) were made up to 0.02 molar concentration in 0.02 molar ammonia solution. (Ammonia was used to neutralise the HCl associated with the amino acid). Similarly 0.02M D-ribose was made up in 0.02M ammonia. Equal aliquots (8 ml) of each solution were mixed in a 50 ml sample bottle and the fluorescence at zero time was determined. The cuvette was closed with a teflon cap and left in the spectrofluorophotometer. The bottle containing the balance of the mixture was sealed with a screw cap and placed in a 37°C oven. A second mixture was made for pH determination. After 3 and 10 days the fluorescence of the samples incubated at room temperature and at 37°C were measured. Furthermore after 10 days, the 37°C sample was diluted 1 to 100 and the fluorescence redetermined.

Anion exchange.

Aliquots of commercial dry gelatine, 120 g, were suspended in 4 l of distilled water and soaked for 30 min. The mixtures were then heated to 40°C in a waterbath with occasional stirring until dissolved.

A 45 mm diameter column was partially filled with 437 ml (Bayer S.A., Isando) Lewatit MP62 macroreticular weak base anion exchange resin and washed with warm ± 60°C distilled water and then fed with the gelatine solution at a rate of 8 to 10 bed volumes per hour using a peristaltic pump. Initially the eluate had a pH of about 8 and this dropped steadily as the amount of liquor treated increased. The treated liquor pH was adjusted to pH 5 to 5.5 with 5N sulphuric acid and then it was concentrated in 4 l lots to about 10% concentration in a vacuum evaporator. The concentrated liquor was filtered through paper pulp, set, cut and dried before grinding to a powder for analysis. The column was used to treat many hundreds of bed volumes of gelatine solution and as the column became exhausted so the pH of the eluate approached the pH of the feed liquor.

Three samples of treated dry gelatine K939, Q939 and F972 were submitted to spectrophotometric and fluorescence analysis for which gelatine 178/12 acted as a control.

After treating some 1200 bed volumes of 3% w/v gelatine solution or 15.7 kg

of gelatine the column was regenerated with 1 l of 5% NaOH solution. The 1 l of regenerant solution collected from the column was very dark. The pH was measured. A 200 ml aliquot required 140 ml N/10 HCl to reduce the pH to 6.0. A 1:50 dilution of the original and the acidified regenerant solutions were examined for fluorescence.

After finding that anion exchange had not affected the fluorescence of gelatine (samples 939 and 972) it was decided to determine whether anion exchange affected the colour of gelatines darkened by reaction with ribose:

A 25 mm diameter column was partly filled with 100 ml Lewatit MP 62 resin. The column was used to treat various (225 g) lots of gelatine solution at a flow rate of 10 to 16 ml/min and a temperature of about 40°C. The absorbance of the gelatine feed to the column and the eluate was determined using the Jenway Colorimeter with filter No 2, and a plastic 1 cm path length cell. In preliminary trials it was found that differences due to different cells made it necessary to use only one cell which was washed three times with aliquots of solution to be tested before a reading was taken. It was noted that absorbance values given by the colorimeter were very different to those given by the Beckman DU 70 spectrophotometer, on the same gelatine.

Gelatine solutions were made using the following formula:

Gelatine	15.0 g
Ribose	6.3 g
Thymol	0.2 g
0.2N NaOH	± 10 ml to pH 6
Water	qs to 225.0 g.

Gelatine solutions with and without ribose were made up in duplicate, in glass jars fitted with screw caps. After soaking for 30 min. and then dissolving at 40°C the sealed glass jars were placed in a 37°C oven. After incubation, the solutions without ribose were cloudy (due to precipitation of salts) hence all the solutions were filtered through Whatman GF/A papers prior to the measurement of their absorbance before anion resin treatment. The remainder of the filtered solution was passed through the anion column and the last 50 ml of eluate was used for measurements of the absorbance after the pHs had been adjusted to 5.0 to 5.5 by adding 3 drops of 5N sulphuric acid. This ensured that absorbance measurements before and after anion treatment were made on solutions of similar pH. The column was washed with warm water between

samples of gelatine.

RESULTS and DISCUSSION.

The detailed results are presented in the Addenda as follows:
Addendum 1. The origins of the gelatines used.
Addendum 2. Detailed analytical data on some of the gelatines used.
Addendum 3. Colour and fluorescence data on all the gelatines used.
Addendum 4. Data on the interaction of gelatine with GLUCOSE.
Addendum 5. Data on the interaction of gelatine and RIBOSE.

A phenomenon encountered working with colloidal gelatine solutions was that the second-order Rayleigh scatter was very pronounced i.e. if the excitation wavelength was 300 nm then the emission spectrum showed a peak at 300 nm due to first order Rayleigh scattering and another at 600 nm due to second-order Rayleigh scatter (Munck and de Francisco, 1989d). Similarly when the excitation wavelength was set to 600 nm a Rayleigh scatter peak was seen at 300 nm.

1. Type B Gelatine fluorescence.

The fluorescence excitation and emission spectra of a pale and a dark Type B gelatine from bovine hide were examined. From the "Multi-spectra" it was clear that between the emission wavelengths of 200 to 700 nm there was significant fluorescence only at excitation wavelengths of about 300 nm and emission wavelengths of about 400 nm. Detailed analysis showed that the excitation maximum was at about 335 nm which gave an emission maximum at 385 nm - see Table 1 below. (Due to quenching a steady, repeatable emission output was not obtained). As could be seen from Figure 4 a pale gelatine (YSA/2). showed a weak emission peak (50 units) at 385 nm and continued lesser emission (49 units) to 410 nm and a dark gelatine INOB/1 (Figure 5) showed a distinct emission peak (169 units) at 385 nm and a second peak (168 units) at 410 nm. (Both emission spectra showed narrow peaks of the same intensity at 360 nm. These Raleigh scatter peaks were caused by the emission scan starting so close to the excitation wavelength of 335nm hence they were ignored).

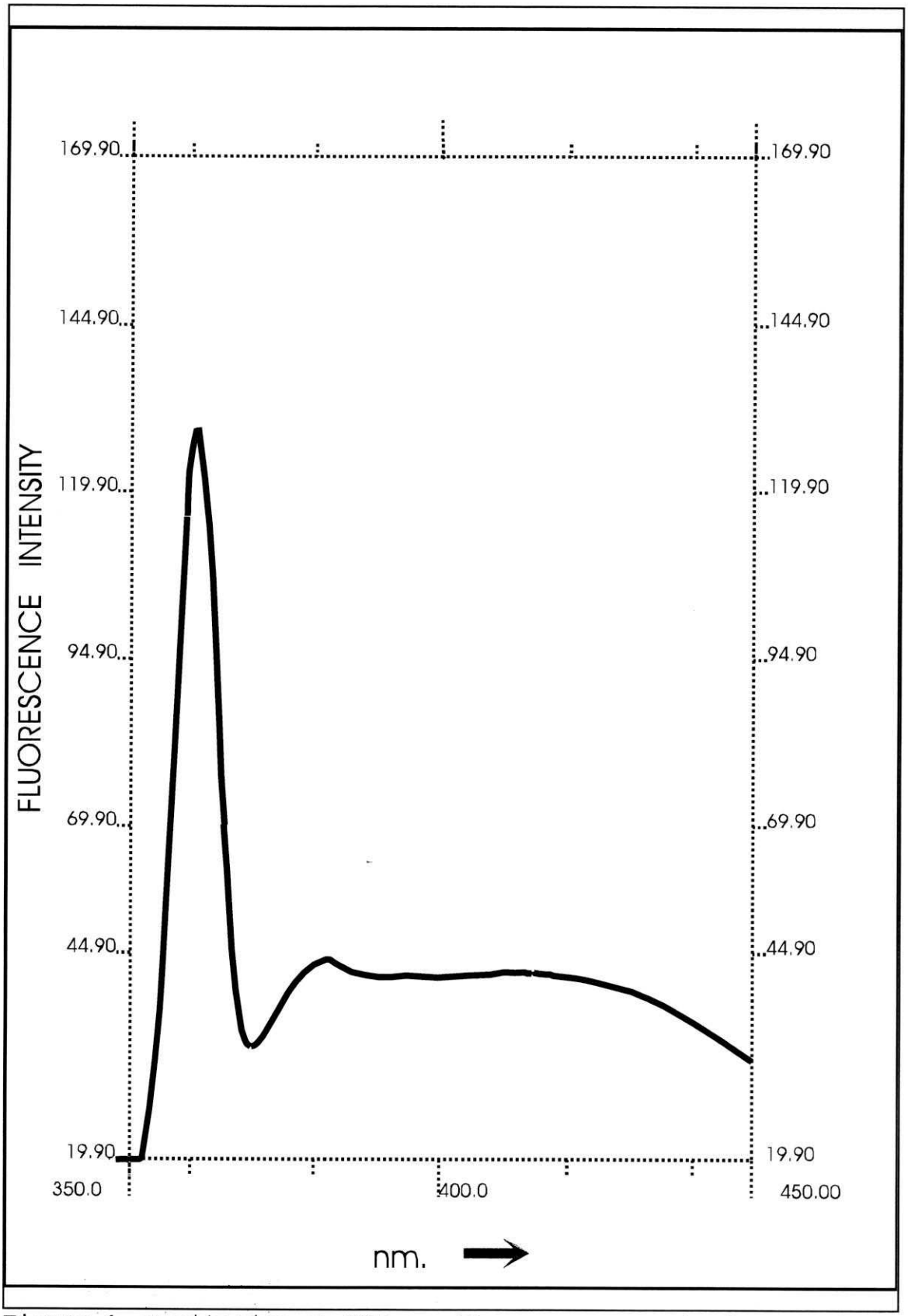


Figure 4. Excitation at 335 nm. Fluorescence emission spectrum of a 1% aqueous solution of a pale (Colour 4.4) Type B gelatine from 10 month old calf skin (YSA/2). Excitation at 335 nm.

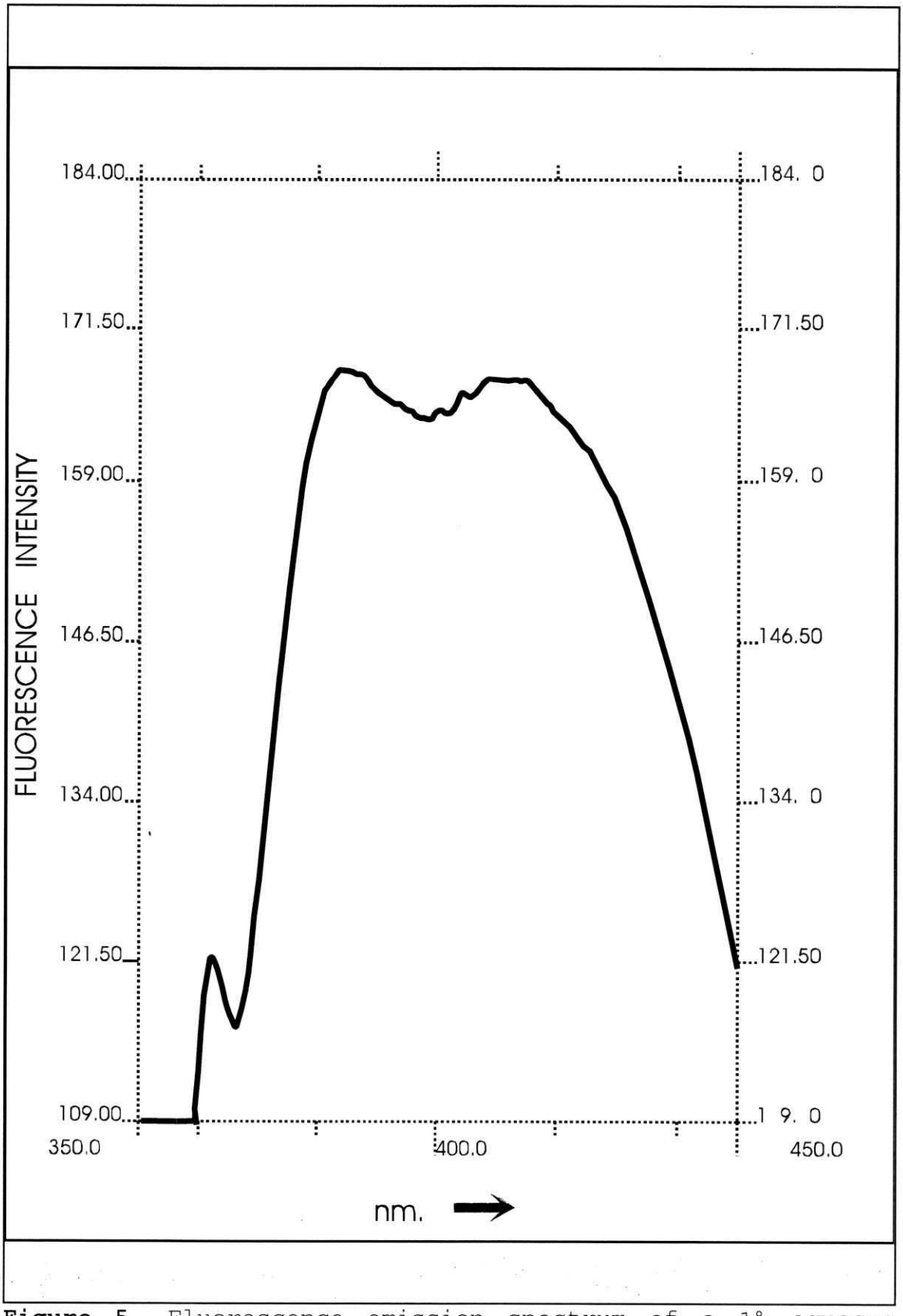


Figure 5. Fluorescence emission spectrum of a 1% aqueous solution of dark (Colour 16.0) Type B gelatine from 12 year old Inguni cow skin (INOB/1). Excitation at 335 nm.

An attempt was made to find (Table 1) the 295/395 pyridinoline cross-link peak reported by Uchiyama *et al.* (1991) for collagen, but this peak was not observed even with the palest gelatine (YSA/1 and YSA/2). Excitation at 295 nm gave emission peaks which bore a striking resemblance to the peaks due to 335 nm excitation only at a slightly lower wavelength, hence they could not be attributed to a different fluorophore. It was concluded that if the pyridinoline cross-link survived the Davis extraction process, its presence would only be detectable after separation from the pentosidine cross-link.

From Addendum 3 it could be seen that linear correlation of all the 335/385 (Maillard) fluorescence data with gelatine colour gave a highly significant correlation coefficient r = 0.78 with 73 degrees of freedom and a correlation equation:

Colour = 0.0066 + 0.0579 x Fluorescence Intensity.

That the value of r was not higher was interpreted as indicating that there was some other factor as well as fluorescence contributing to gelatine colour.

In summary:

Type B gelatine made by the lime-sulphide alkali conditioning process followed by sulphurous acid acidulation had an excitation maximum at about 335 nm and emission maxima at 385 nm and about 410 nm. Both these maxima became stronger and more pronounced with increasing animal age hence it would appear that both were associated with Maillard cross-linking. From the investigations of Sell and Monnier (1989) it was evident that the reaction of lysine, arginine and ribose had not given only one product and from this study it would appear that there could be at least two forms of the Maillard cross-link with similar but not identical structure.

Table 1. *Gelatine fluorescence emission peak data for excitation at 335 and 295nm.*

Sample	Visual Colour	Excitation at 335 nm				Excitation at 295 nm			
		Peak 1		Peak 2		Peak 1		Peak 2	
		nm	Height	nm	Height	nm	Height	nm	Heigh
6Y4B/3	9.5	385	174	410	172	383	106	412	99
6Y4B/2	8.0	387	164	407	160	385	114	-	-
6Y4B/1	6.4	385	183	415	185	380	104	418	98
CT6/2	5.6	388	144	412	147	381	89	410	81
CT6/3	6.0	385	148	415	154	381	88	410	87
3Y4A/1	6.6	385	102	405	99	381	73	-	-
5Y6A/1	6.4	385	118	410	118	378	73	-	-
5Y6A/3	9.4	385	158	415	166	378	90	423	87
5Y6A/2	8.4	385	134	410	139	378	86	410	77
CT6/1	5.2	385	115	-	116	379	83	-	-
3Y4/2A	6.0	385	138	-	-	389	70	405	62
YSA/2	4.4	385	50	410	49	361	34	410	30
YSA/1	3.6	382	41	412	40	361	32	410	25
YSA/3	4.8	385	75	-	-	378	43	410	39
5Y4/3	8.4	385	151	410	156	380	86	420	83
5Y4/2	6.8	385	135	410	136	382	77	415	70
IOB/1	10.0	385	169	410	168	382	112	408	104
IOB/2	16.0	385	207	410	201	385	112	415	110
IOB/3	20.0	385	254	410	249	385	127	428	132
AVERAGE		385.1		410.7		378.9		413.6	

The analytical data on each sample is recorded in Addendum 3.
- indicates that the data could not be read from the graphs.

2. Type A calf skin gelatine.

Tanaka et al. (1988) showed a ribose induced 370/440 nm fluorescence in collagen. In this study there was one instance of this fluorescence occurring naturally and that was with Calf skin Type A gelatines where the 370/440 fluorescence intensity of 121 was many times stronger than the pentosidine

335/385 nm fluorescence of 21. It was observed that the excitation spectrum of gelatine Calf-A/1 monitored at 385 nm (Figure 7) had peaks of 24 units and 21 units at about 330 nm and 290 nm. When monitored at 440 nm there was very much stronger excitation peak (125 units) at 370 nm (Figure 6). For excitation at 335 nm the emission spectrum shows two clear peaks of 22 units at ± 380 nm and 65 units at 440 nm (Figure 8).

The 290 nm excitation maximum in Figure 7 was significant because:

1. This was the only instance of an excitation peak at this wavelength. According to Uchiyama *et al.* (1991) this fluorescence was characteristic of the pyridinoline cross-link and according to Eyre (1987) this cross-link was not found in skin.

2. From the work of Uchiyama *et al.* (1991) it was clear that this fluorescence was caused by the presence of the pyridinoline cross-link which was found here in Type A bovine gelatine but not in Type B gelatine.

It was concluded that both the 295/395 and the 370/440 fluorophores only found in Type A calf skin gelatine were destroyed by lime-sulphide conditioning followed by sulphurous acid acidulation of the collagen but they were not affected by sulphuric acid treatment for the production of Type A gelatine.

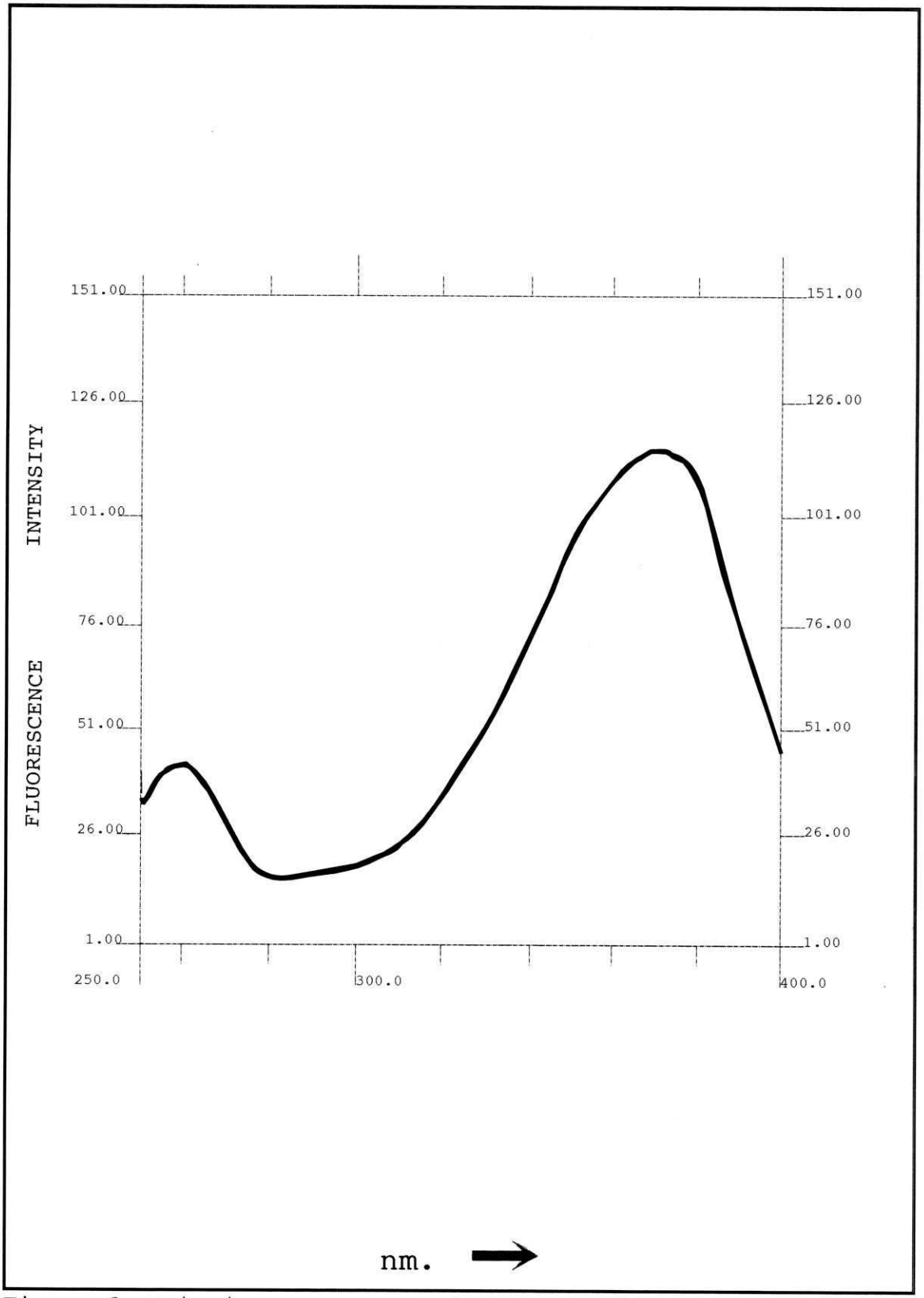


Figure 6. Emission at 440 nm. Fluorescence excitation spectrum of a 1% aqueous solution of Type A calf skin gelatine (Calf Type A/1). Note the excitation maximum at 370 nm.

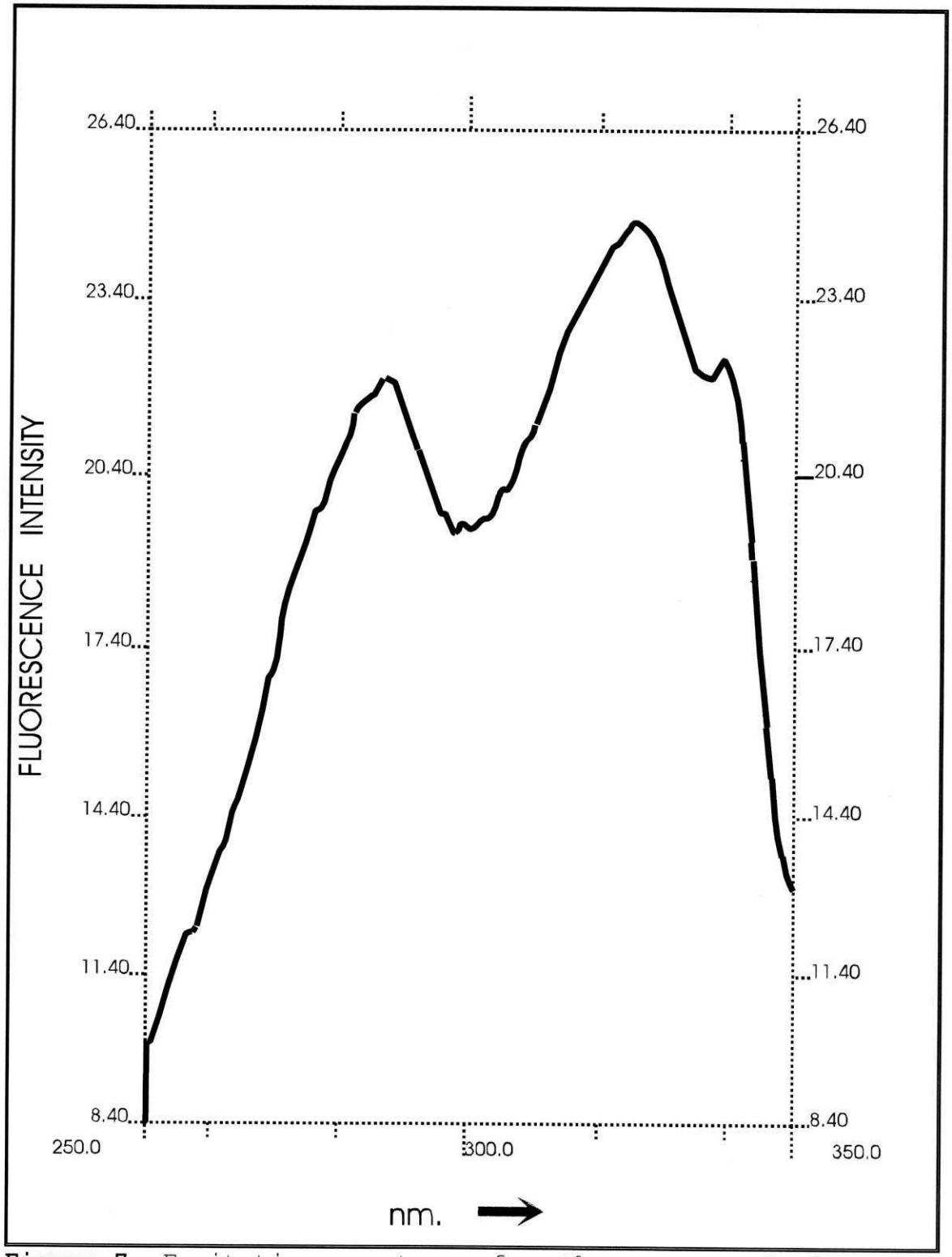


Figure 7. Excitation spectrum of a 1% aqueous solution of a Type A calf skin gelatine. Note - excitation maxima at 290-295 nm and at 335 nm. Emission at 385 nm.

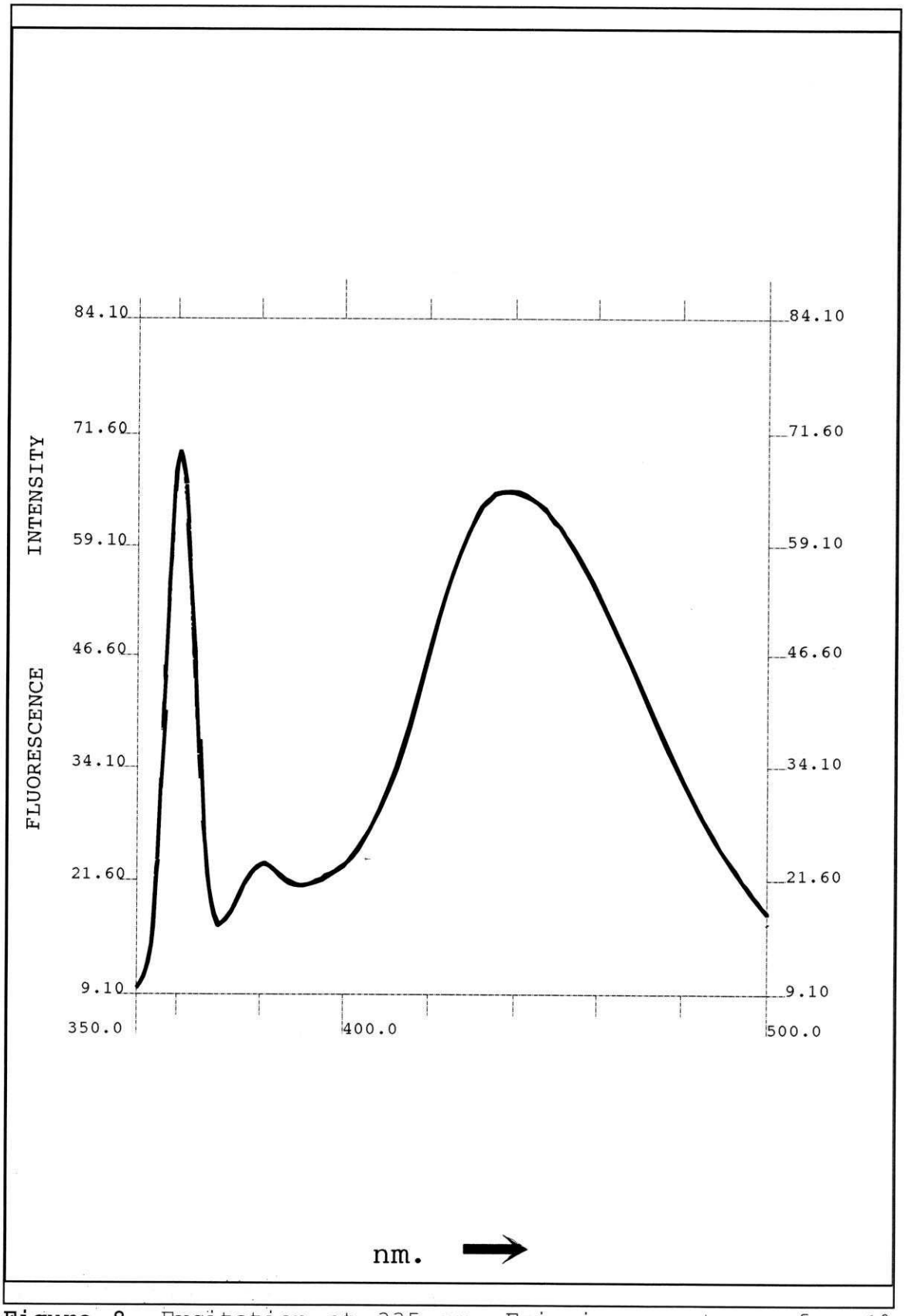


Figure 8. Excitation at 335 nm. Emission spectrum of a 1% aqueous solution of Type A calf skin gelatine. Note - emission maxima at 385 and 440 nm.

3. Pigskin gelatine.

Type A pigskin gelatine was expected to be similar to calf skin Type A gelatine, however, when pigskin gelatines were scanned only one excitation maximum was found at 335 nm (Figure 9) but these gelatines had a stronger emission peak at 420 nm than at 385 nm with only a shoulder to indicate that there was a peak at 385 nm as shown in Figure 10. i.e. the Maillard 335/385 fluorescence seems to be more marked in bovines than in pigs, however the pigskin gelatines (designated D...) had shown a significant (0.005) correlation between colour and pentosidine fluorescence ($r = 0.895$).

4. Fish skin gelatine.

The two fish skin gelatines made by the acid pigskin process also had a stronger fluorescence at 420 nm than at 385 nm but there was no indication of a relationship between colour and pentosidine fluorescence (see Addendum 3.)

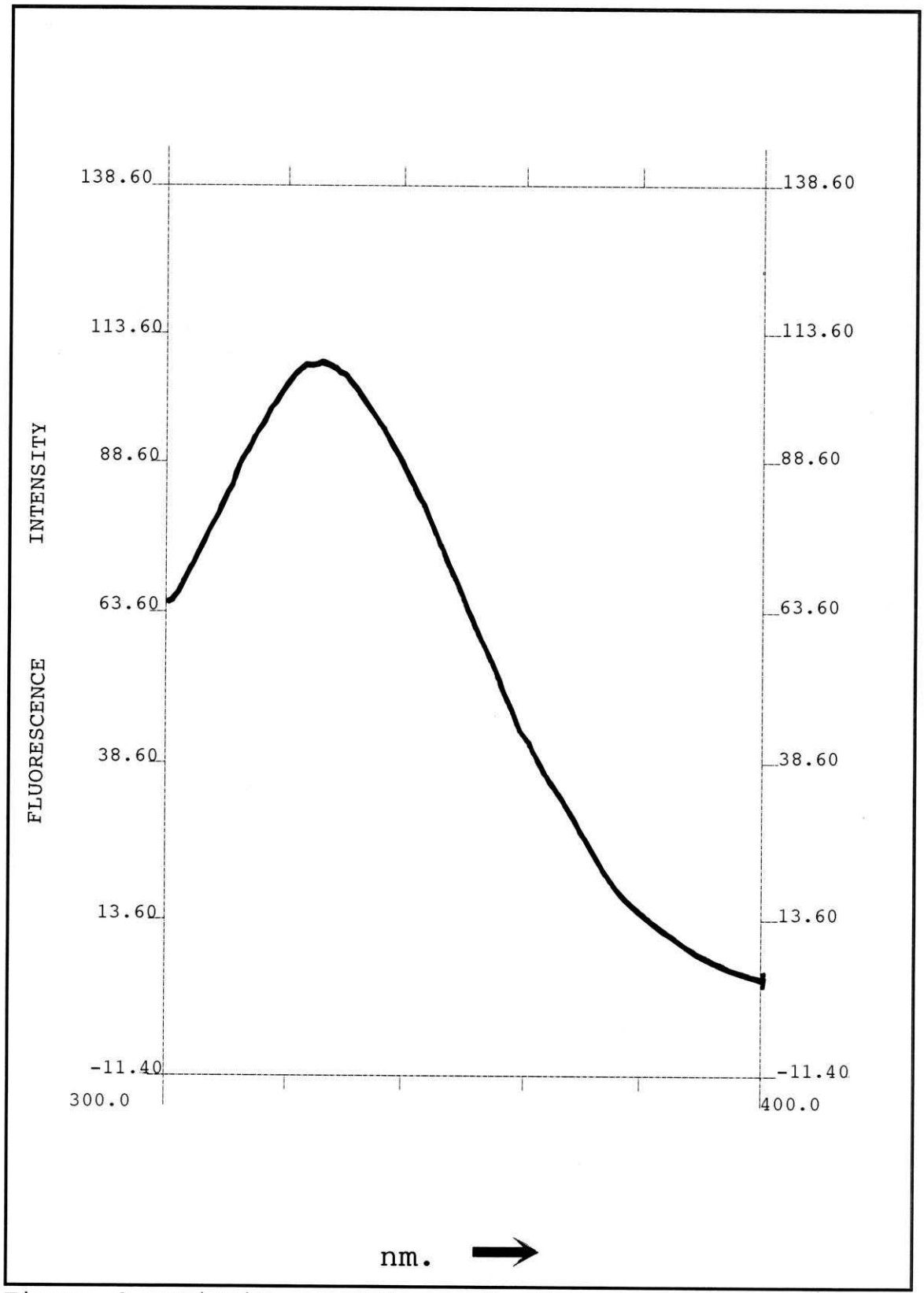


Figure 9. Emission at 420 nm. Excitation spectrum of a 1% aqueous solution of pigskin gelatine (D1512). Note excitation maximum at ± 330 nm.

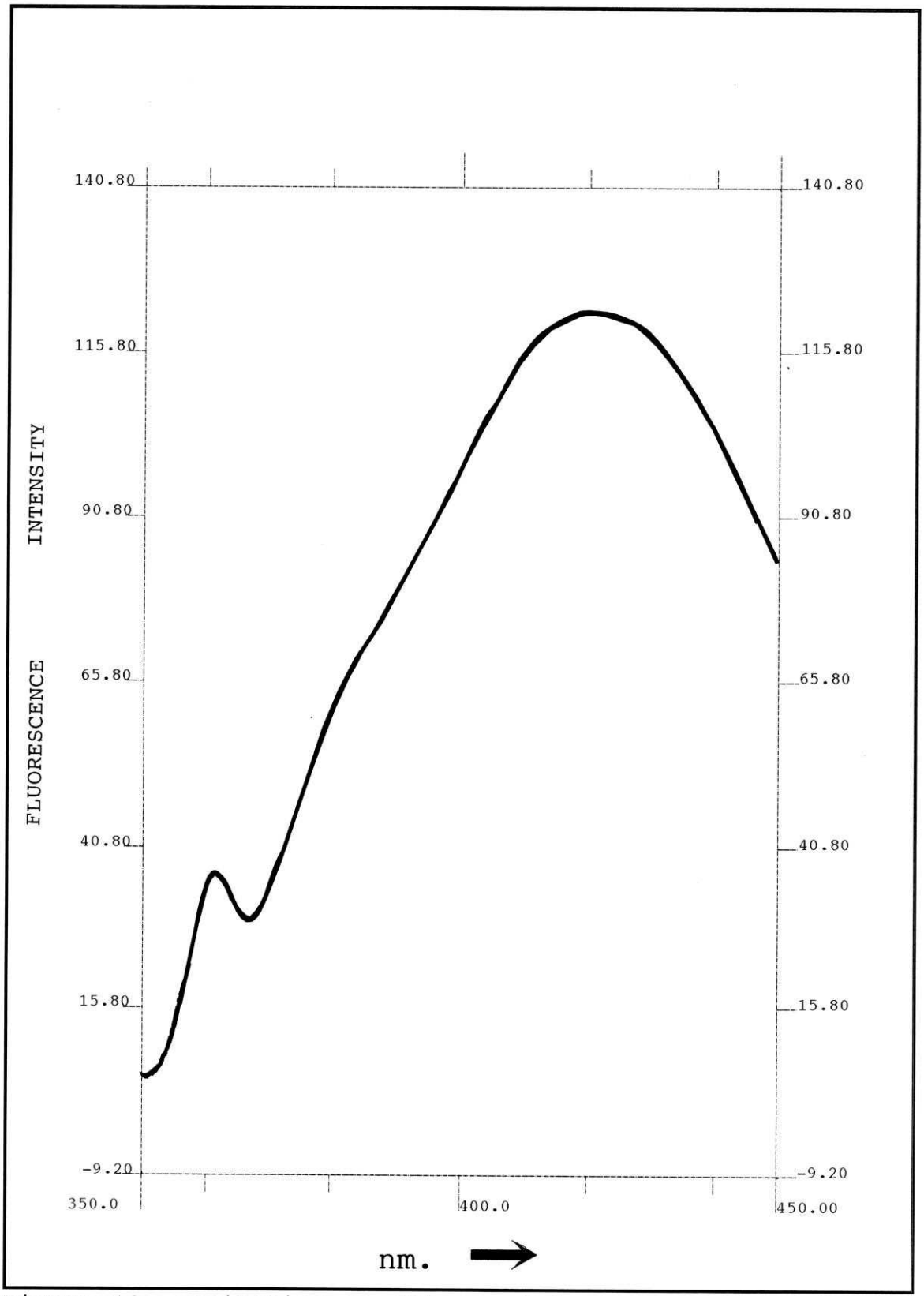


Figure 10. Excitation at 335 nm. Emission spectrum of a 1% aqueous solution of pigskin gelatine. Note shoulder at ± 385 nm and maximum at 410 nm.

Table 2. *Colour and fluorescence data for gelatines derived from animals of known age.*

SAMPLEϕ	ANIMAL AGE MONTHS	OVERALL COLOUR	% EXTRACTABILITY						COLOUR in Davis Units.			Fluorescence Emission Intensity - Excitation @ 335 nm.		
			45°C	50°C	55°C	60°C	70°C	3 RUNS	1ST	2ND	3RD	1ST	2ND	3RD
YSA	10	4.3	35	26	20	-	-	83	3.6	4.4	4.8	41	50	75
CT6	18	6.4	40	32	23	-	-	95	5.2	5.6	6.0	115	144	148
5Y4	58	7.2	20	21	-	-	-	70	5.6	6.8	8.4	104	126	140
5Y4	58	7.2	20	21	-	-	-	70	5.6	6.8	8.4	94	129	142
5Y6A	58	8.2	21	21	-	-	-	72	6.4	8.4	9.4	118	134	158
5Y6A	58	8.2	21	21	-	-	-	72	6.4	8.4	9.4	115	128	148
ST12	144	18.1	4	7	-	-	-	29	6.4	10.0	10.7	105	124	167
ST16	144	13.1	12	17	-	-	-	55	10.7	12.3	14.5	174	192	228
INOB	143	(21.7)		9	-	20	27	57	16.0	16.0	20.0	169	207	254
6Y4B	78	7.3	17		21	33	-	71	6.4	8.0	9.5	183	164	174
YSA*	10	4.3	35	26	20	-	-	83	3.6	4.4	4.8	38	48	68
CT4*	18	6.4	23	28	28	-	-	76	5.6	5.6	5.6	104	133	142
5Y4*	58	7.2	20	21	-	29	-	70	5.6	6.8	8.4	104	126	149
ST24*	144	15.0	10	13	-	26	-	49	8.9	12.3	13.3	133	168	214
WT4*	144	16.3	4	8	-	17	-	29	11.4	12.3	13.3	114	130	172
INOE*	143	14.9		11	-	23	37	72	9.4	12.3	16.0	175	207	NA
CTO4*	156	16.4	10	11	14	-	-	35	10.0	9.4	10.0	187	223	239
3Y4A*	40	5.2	28	30	-	-	-	-	6.6	6.0	-	102	138	-
6Y4A*	78	7.9	15	21	-	34	-	70	8.4	7.6	8.9	141	165	174

ϕ See Addendum 2 for details of origins etc.
* Samples had equal conditioning - 4 weeks, except for YSA.
() Not included in the correlation due to process differences.
- Not applicable.

Table 2 contains the colour and fluorescence data for the gelatines extracted from the skin of animals of known age enabling statistical analysis for linear correlations between animal age, colour and fluorescence.

Correlations:

1. Animal Age & Overall Colour.

   Samples *. $r = 0.976$, $df = 6$, Sig. > 0.01

   Samples All. $r = 0.953$, $df = 15$, Sig. > 0.01

2. 45°C Extractability & 1st Colour.

   Samples *. $r = -0.933$, $df = 5$, Sig. > 0.01

   Samples All $r = -0.779$, $df = 14$, Sig. > 0.01

3. 50°C Extractability & 2nd Colour.
   Samples *. r = -0.915, df = 5, Sig. > 0.01
   Samples All r = -0.816, df = 9, Sig. > 0.01

4. 1st Colour & 1st Fluorescence.
   Samples All. r = 0.6934, df = 15, Sig. > 0.01

5. 2nd colour & 2nd Fluorescence.
   Samples All. r = 0.6979, df = 16, Sig. > 0.01

6. 3rd Colour & 3rd Fluorescence.
   Samples All. r = 0.3852, df = 14, Not Significant.

The above shows that there were significant correlations between animal age and overall colour, between gelatine 1st and 2nd run extractability and colour, and between 1st and 2nd extraction gelatine colour and pentosidine fluorescence intensity. In other words, the Maillard cross-linking as determined by fluorescence intensity affected hide extractability and gelatine colour similarly - a not unexpected observation, and it did confirm that, to an extent, colour was a part of the protein and not a removable contaminant.

EFFECT OF INCUBATING Gelatines WITH GLUCOSE.

This was the first experiment of this type that was conducted, hence it showed some of the characteristics of a preliminary experiment in that experimental determinations were added after the start of the experiment to analyse or explain the emerging data.

The detailed results of the monitoring of the colour, fluorescence and pH of the gelatine-glucose solutions with starting pHs of 6 and 8.75 are given in ADDENDUM 4. These results are summarised in Figure 11.

Glucose gelatine interaction - Effect on Colour.

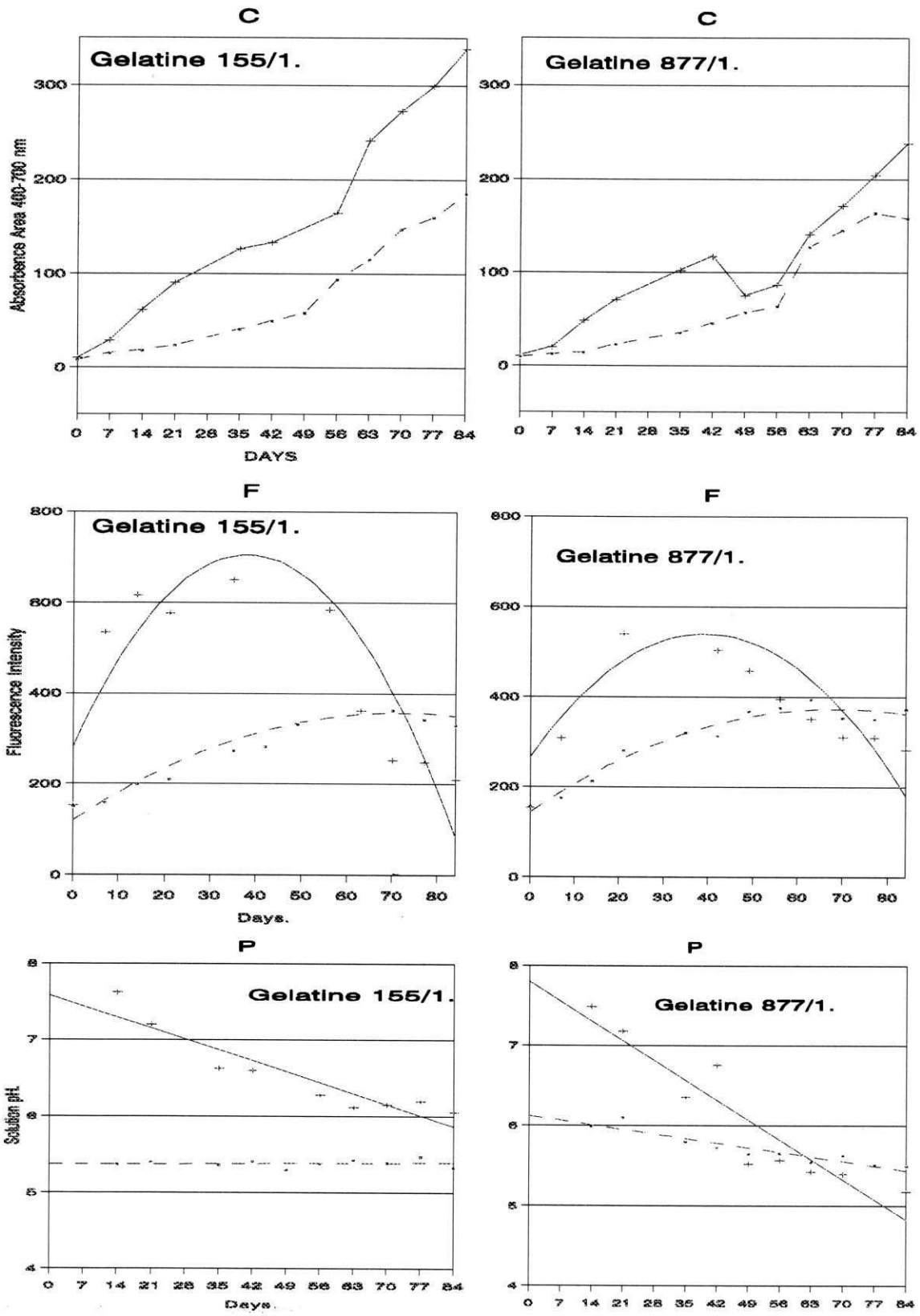
In the previous section it was shown that the Gardner instrument gave the best correlation between instrumental and visual colour measurements. The Gardner instrument was however not available for this series of experiments, hence, the alternative colour measurement using a spectrophotometer and measuring the area under the curve between 400 nm and 700 nm had to be used.

The apparent "lag phase" to the development of colour in the gelatine-glucose solutions, particularly with the samples starting at pH6 was noted. It was also apparent that the starting pH of 6 resulted in an overall lesser development of colour than with a starting pH of 8.75.

A comparison of the absorbance spectra of a naturally pale gelatine 155/1 and a naturally dark gelatine 178/12 and glucose darkened gelatines 155/1 and 877/1 are shown in Figure 12. These spectra show a smooth increase in absorbance from the light 155/1 to the darkest 178/12 over the whole spectral range which would tend to confirm the contention that pentosidine and gelatine colour originate from glucose *in vivo* (Dyer *et al.*, 1991).

Finally, the two gelatines 155/1 and 877/1 behaved similarly but not identically. After 84 days gelatine 877/2 and 155/1 had produced similar amounts of colour at pH 6 as evidenced by the 400 nm absorbances of 2.11 and 2.36, but at pH 9 the colour produced by gelatine 155/1 was an order of magnitude greater (with a 400 nm absorbance of 4.68) than was the colour produced by gelatine 877/1 (with a 400 nm absorbance of 3.01).

It was also noteworthy that the 400 nm absorbance was, as with colour, closely related to the area under the absorbance curve between 400 and 700 nm (r=0.999) as shown in Table 3.



The effects of gelatine glucose interaction on (C) colour, (F) fluorescence intensity and (P) pH at starting — pH 8.75 and - - pH 6. Gelatines 155/1 & 877/1 had similar gel strength and colour.

Figure 11. Gelatine glucose interaction. (10% w/w gelatine in 0.2M aqueous glucose solution incubated at 50°C).

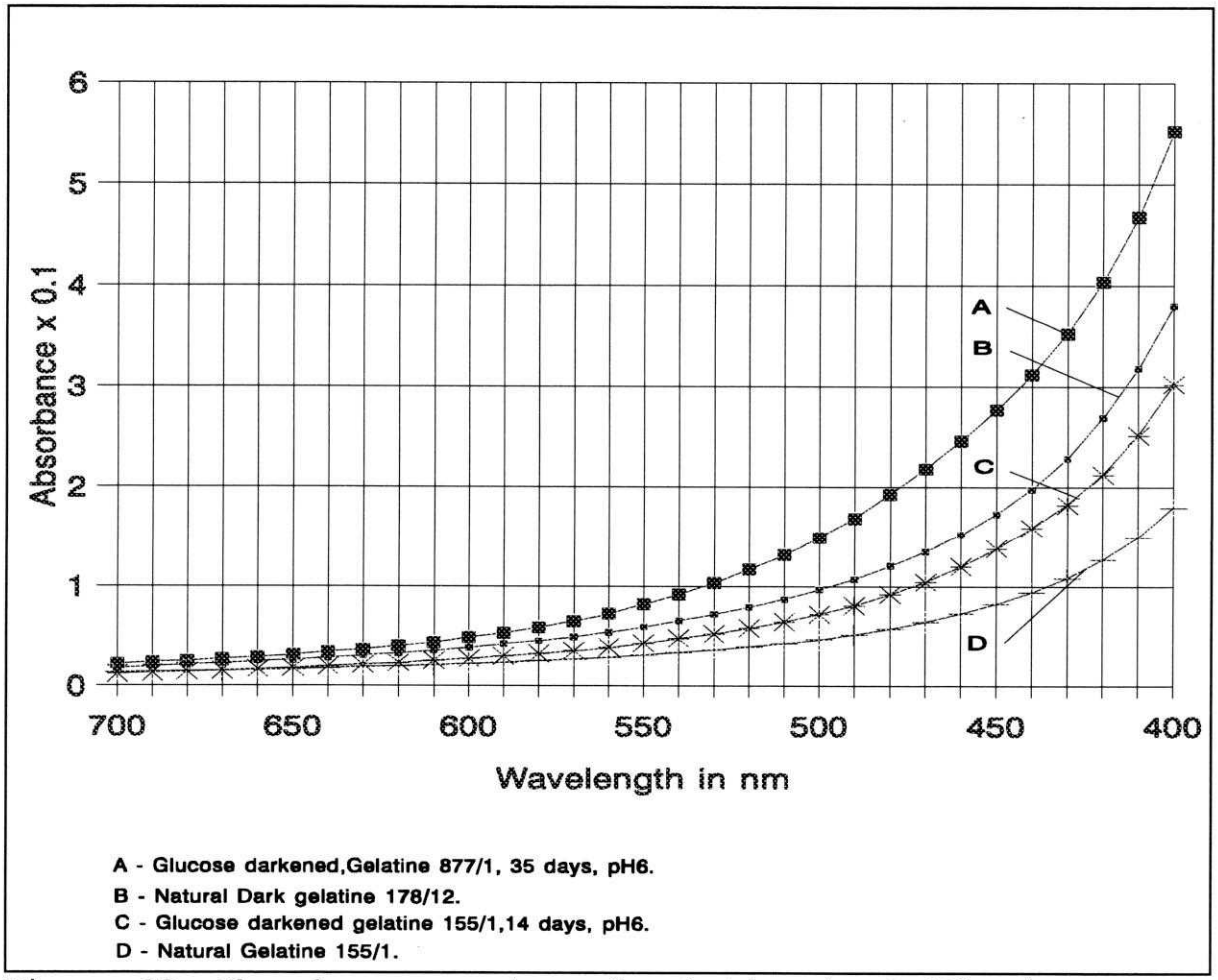


Figure 12. Absorbance spectra of gelatine in 6.67% w/v aqueous solution. Glucose darkened by incubating 10% w/w gelatine in aqueous glucose solution (0.2M) at 50°C.

Table 3. *A comparison of the area under the absorbance curve between 400 and 700 nm and the absorbance at 400 nm for glucose darkened gelatine 155/1 starting at pH 9.*

Absorbance at 400 nm	Curve Area 400 to 700 nm.	Absorbance at 400 nm	Curve Area 400 to 700 nm.
0.18	10	0.5	28
0.94	61	1.35	90
1.89	126	2.31	164
3.74	272	4.68	339

Absorbances of > 4.7 were outside the range of the instrument.
see ADDENDUM 4, Sample B.

Glucose gelatine interaction - Effect on Fluorescence.

The increase in 335/385 nm fluorescence intensity of the gelatine-glucose solutions with time was evident from Figure 11. As with colour, there was a markedly greater production of fluorophore in solutions with a starting pH of 8.75 than in solutions with a starting pH of 6.

The eventual decrease in fluorescence intensity with time, which was particularly noticeable with the solutions starting at pH 8.75, was typical of concentration quenching (Munck and de Francisco, 1989e). From the continuously increasing fluorescence intensities of the 0.01% gelatine solutions it was concluded that concentration quenching was the reason for these observations.

In Figure 11 the fluorescence v/s time data is shown as curves derived using second order polynomial regression with the following correlation coefficients (n = 12):

Gelatine 155/1 at pH 6. r = 0.94
Gelatine 155/1 at pH 8.75. r = 0.81
Gelatine 877/1 at pH 6. r = 0.97
Gelatine 877/1 at pH 8.75. r = 0.77

Tanaka et al. (1988) noted 370/445 nm fluorescence in ribose treated collagen. Hence, these wavelengths were also monitored. After 8 weeks, no excitation maximum other than that at about 350 nm could be observed, as shown in Figure 13. Hence, the data for these wavelengths has not been recorded. The apparent movement of the excitation maximum from 335 nm was due to observation of the emission at 420 nm instead of 385 nm.

Glucose gelatine interaction - Effect on pH.

It is evident from Figure 11. that there were marked changes in the pH of the solutions with time. A change in solution pH would be expected to result from the reaction of glucose with the amine side chains of lysine and arginine but such a pH change would be expected to be smooth and consistent even if not linear. From the graphs it is evident that the changes in solution pH were somewhat erratic. For example gelatine 155/1 starting at pH 8.75 had, by day 49, a pH of 5.40, whereas at day 56 the pH was 6.27. It was concluded that

this variance could only be attributed to experimental error. As the pHs of the daily standards were constant it appeared that this error had to be due to inadequate mixing after pH adjustment at the start of the experiment (possibly due to the onset of gelation). Similarly the variances in the gelatine 877/1 pHs (starting at pH 8.75) indicate that there was also considerable "experimental error" associated with the results. Measurement of solution pH was not part of the original experimental plan, hence it was not easy to show inconsistencies in pH resulting in inconsistencies in fluorescence or colour, however, the fluorescence maximum for gelatine 877/1 at 35 days coincided with an anomalously low solution pH. This high fluorescence value could be the result of a lower concentration of fluorophore resulting in less quenching.

Glucose gelatine interaction - General observations.

As a general observation it was noted that after about 28 days at 50°C the solutions in the Mc Cartney bottles removed from the incubator had not geled at room temperature. In addition, there was no sign of gelation of the incubated samples as was observed with the ribose interaction. From experience, this was probably due to the effects of thermal degradation at 50°C being greater than those of cross-linking. There was a possibility that the loss of gelling properties could have been due also to microbiological activity. Three of the four controls, after 61 days, (samples F, G, H) exhibited cloudiness. However, these samples were not malodorous, so the cloudiness could have been due also to thermal degradation of the gelatine resulting in precipitation of $CaSO_4$ ash.

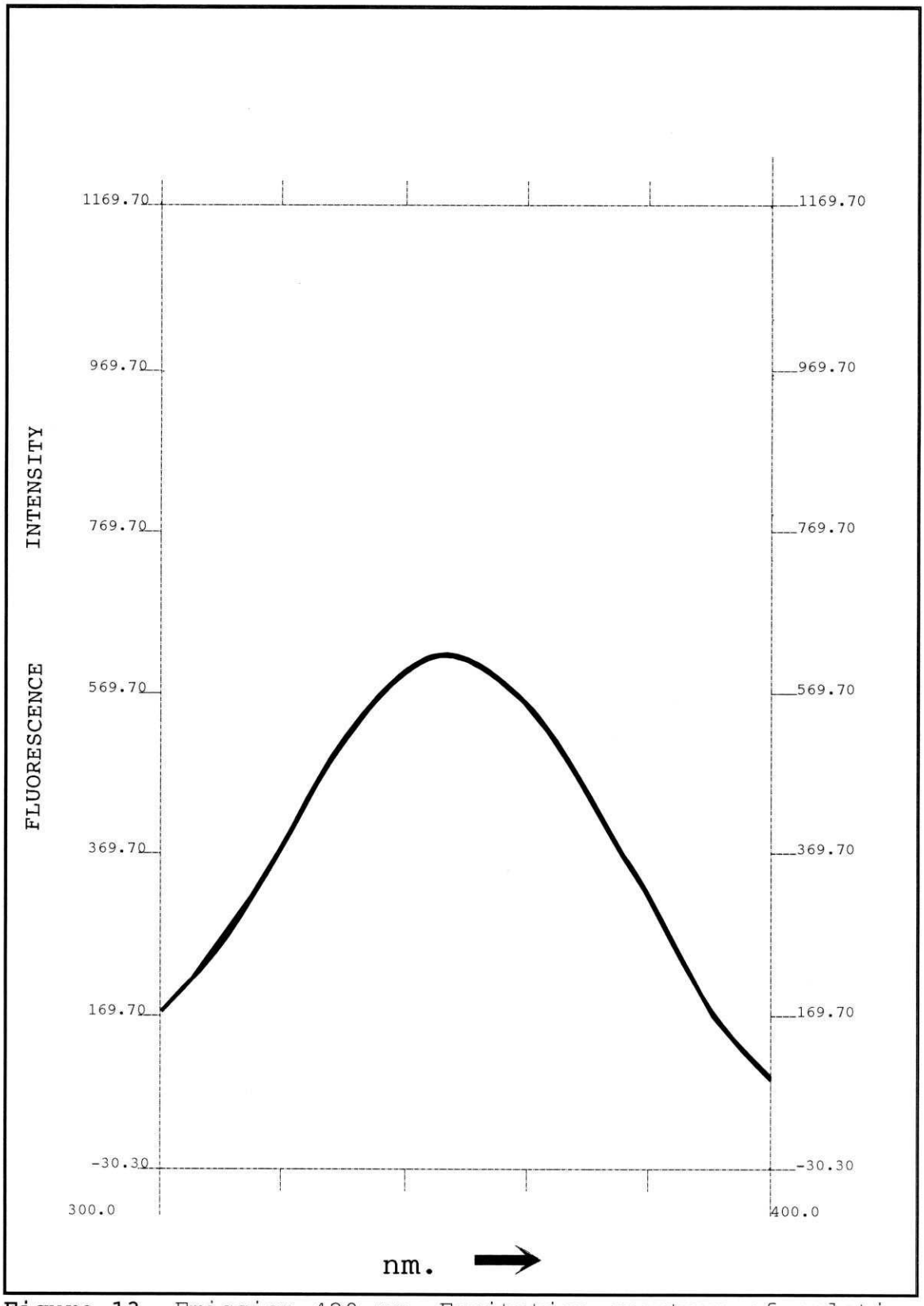


Figure 13. Emission 420 nm. Excitation spectrum of gelatine (155/1) after darkening with glucose for 56 days at 50°C (initial pH = 6.0)

Glucose gelatine interaction - Controls.

As can be seen from the data on the Controls in ADDENDUM 4, Gelatine solutions at pH 6 and 8.75 showed a small increase in colour or area under the absorbance curve of the order of 2 absorbance units at the start of incubation at 50°C whereafter the colour increased slowly by an additional 4 units of absorbance over some 60 days of incubation. In contrast the glucose treated gelatine increased in area under the absorbance curve by some 150 to 300 units, hence, this large increase could not be due to "natural" darkening under the experimental conditions. In other words some 1% of the darkening observed with the glucose - gelatine interaction could have been due to the "natural" darkening of gelatine when it was incubated under the same conditions. It was also noteworthy that whereas glucose treated gelatine exhibited more darkening in the solutions starting at pH 8.75 than those starting at pH 6, the reverse was observed in the absence of glucose.

The fluorescence intensity of the controls increased very slightly with time of incubation and solution pH decreased very slowly with time. These results could be interpreted as indicating that the gelatine contained a small amount of Amadori reaction products or sugar which could continue with the Maillard reaction in solution yielding a small amount of colour and fluorescence. However, gelatine hydrolysis at 50°C would be expected to also contribute to variance in pH.

Glucose caramelization.

For the sake of completion it was decided to establish whether glucose alone underwent any significant changes caused by the experimental conditions. From Table 4 it is clear that there was no caramelization of glucose. The apparent change in fluorescence intensity was well within the limits of experimental error and therefore negligible. The glucose solution pHs were 6.47 and 7.87, respectively, at the end of the test period. The differences from the starting pHs were noted but this did not affect the conclusions that glucose alone had not changed colour or degree of fluorescence under the conditions of the experiment.

Table 4. *The effect of 50°C incubation on a 3.6% glucose solution, at pH 6 and pH 8.75, on the colour and fluorescence intensity of the solutions.*

	FLUORESCENCE INTENSITY			
WEEK No.	0	6	9	14
pH 6	2.0	1.4	4.2	3.0
pH 8.75	2.0	1.4	2.4	3.0
	C O L O U R			
WEEK No.	0	6	9	14
pH 6	1.0	0.8	0.9	0.5
pH 8.75	0.9	0.7	0.8	0.4

THE EFFECT OF INCUBATING GELATINE WITH RIBOSE.

Ribose is a pentose sugar, hence it forms a ring structure that is less stable than the gluco-pyranose ring structure and for this reason, the pentoses had a greater proportion of molecules in the reactive aldehyde form which would presumably account for the greater Maillard reactivity of ribose (Tanaka *et al.*, 1988).

The reasons for choosing pH 9 for use in the preliminary study No1 was because it was known that the reaction of dilute gelatine solution with formaldehyde, in the Formol titration, was conducted at pH 9 because the reaction with formaldehyde was instantaneous at this pH (Cobbet *et al.*, 1963). Also, from experience in the cross-linking of gelatine with formaldehyde (to form an ether cross-link) it was known that the reaction was very fast at pH 9 while at pH 5 the mixture takes many hours before forming an insoluble gel. Hence, the Maillard reaction with sugar aldehyde would probably be facilitated or accelerated at pH 9.

Preliminary study No 1.

Table 5. *The changes in absorbance of gelatine treated with reducing sugars monitored using a Colorimeter and filter No. 2 (470 nm).*

Treatment	Weeks at 44°C	Absorbance.	
		pH5	pH9
Blank	1	0.097	0.089
Glucose	1	0.103	0.101
Ribose	1	0.146	0.176
Blank	2	0.137	0.145 *
Glucose	2	0.131	0.122 ϕ
Ribose	2	0.417	0.289

* Solution became cloudy with a precipitate probably due to bacterial growth.
ϕ Solution only just gelled at 17°C. All other solutions were strongly gelled at 17°C after two weeks at 44°C.

From Table 5 it was concluded that:

(i) Ribose caused marked darkening of gelatine while glucose did not appear to react. This was in line with common practice of using glucose in gelatine confectionery.

(ii) Although pH 9 seemed to accelerate the rate of colour production with ribose after one week's incubation. this trend seemed to be reversed after incubation for two weeks.

(iii) It was noted that the use of aluminium caps on test tubes did not prevent evaporation which could account for the darkening of the blank after two weeks incubation.

Table 6. *The absorbance characteristics of a pale and a dark gelatine for comparison with the colour of ribose treated gelatine.*

Filter nm.	GELATINE			
	Colour = 8	Colour = 20	Ribose treated @ pH 5 *	Ribose treated @ pH 9 *
400	0.080	0.145	0.347	0.266
470	0.086	0.153	0.417	0.289
490	0.074	0.130	0.328	0.236
520	0.058	0.103	0.220	0.174
540	0.048	0.081	0.149	0.129
580	0.039	o.064	0.106	0.097
600	0.031	0.049	0.070	0.065
710	0.017	0.034	0.041	0.037

*Colour 8 gelatine treated with ribose for 2 weeks at 44°C.

From Table 6 it was concluded that the colour production due to the Maillard reaction had absorbance characteristics similar to that of ordinary dark gelatine. It was evident also that colours darker than Davis Colour 20 were quite possible.

Preliminary study No 2.

Table 7. *The effect of ribose treatment on the physical properties of gelatine at pH 5 and 8.*

Property	Solution 1. pH 5.			Solution 2. pH 8.		
	Days at 37°C.			Days at 37°C		
	0	3	6	0	3	6
Bloom	233	195	188	229	170	168
Colour	7.6	8.9	26.7	11.4	22.8	>32
Clarity	11.1	11.8	12.5	11.8	12.5	13.3
pH	5.5	5.3	5.3	8.4	7.9	7.5
Viscosity	34	33	31	39	44	48
Absorbance with Filter No.						
1	0.058	0.076	0.263	0.068	0.256	0.433
2	0.061	0.080	0.277	0.073	0.295	0.539
3	0.050	0.067	0.231	0.060	0.255	0.492
4	0.041	0.052	0.179	0.041	0.180	0.358
5	0.032	0.039	0.140	0.029	0.108	0.219
6	0.022	0.031	0.117	0.020	0.065	0.133
7	0.016	0.026	0.097	0.010	0.039	0.071
8	0.010	0.015	0.080	0.008	0.019	0.035

From Table 7 it was observed that there was:

1. Markedly faster darkening at pH 8 than at pH 5.
2. Increasing viscosity with time indicating cross-linking at pH 8.
3. A drop in pH with incubation time for the pH 8 sample. The source of acid was not evident, however, the removal of amine side chains due to the Maillard reaction would be expected to have had the same effect.
4. Such a fast reaction at pH 8 that there was some darkening during the time required for the Bloom determination, as evidenced by Solution 2 with a colour of 11.4 at time 0. Solution 1 had a colour of 7.6 at the same time and the original colour of the gelatine was 8.6.

5. A large change in the short wavelength absorbance, using the No 2 filter, due to (the Maillard) reaction but there was relatively little change in the No 8 filter long wavelength absorbance.

Detailed study - Overview.

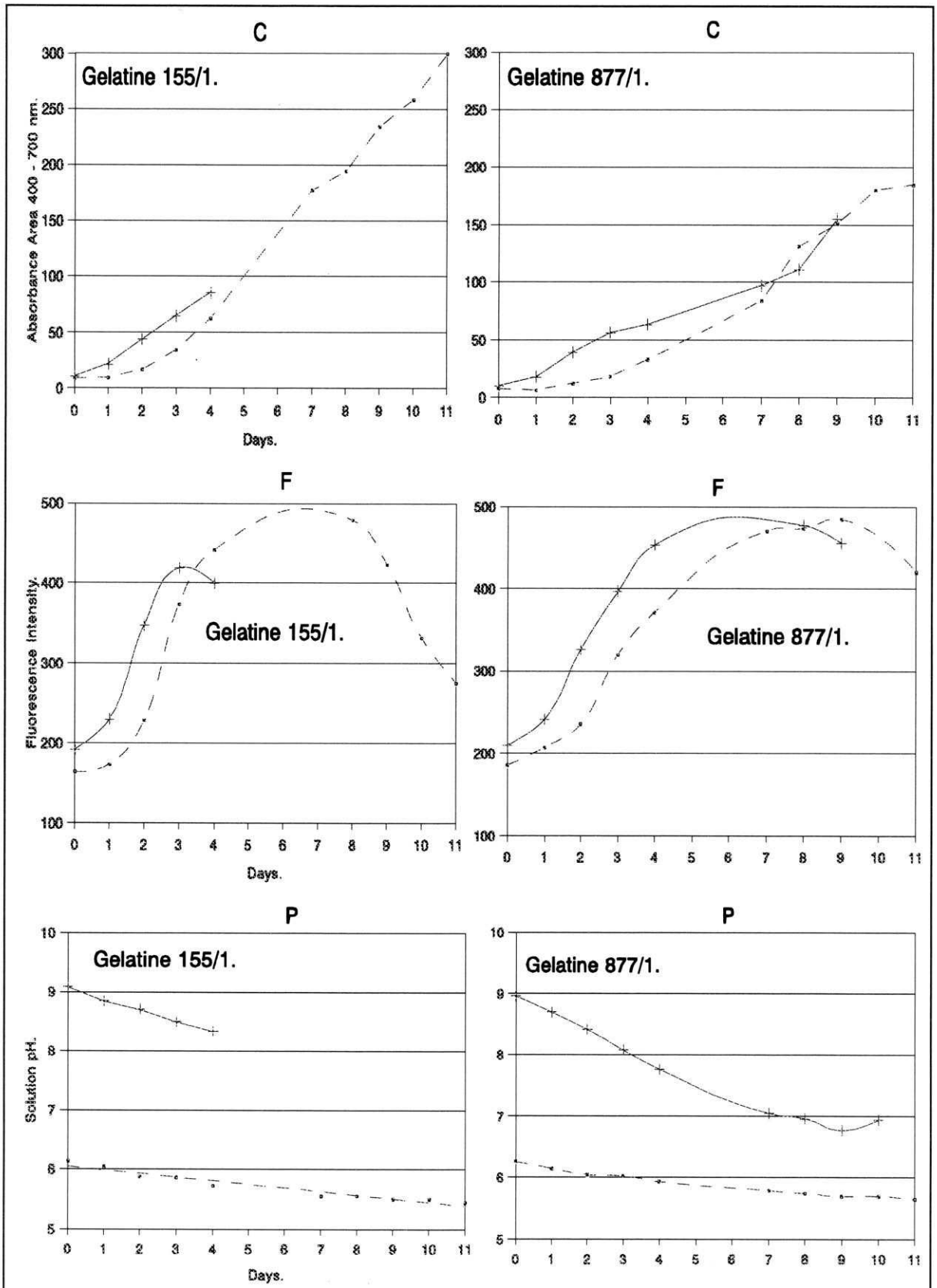
In this study gelatine and ribose were incubated at 37°C and at pH 6 and 9 for up to 12 days with controls. The solution colour, pH, and fluorescence were monitored. The detailed data is given in ADDENDUM 5, and Figure 14 shows the most pertinent results.

Ribose gelatine interaction - Gelation.

All four of the ribose treated gelatines gelled during the course of the trial. Gelatine 155/1 at pH 9 gelled between the 4th and 7th days. Gelatine 877/1 at pH 9 gelled on the 10th day and both gelatines at pH 6 gelled between the 11th and 14th days. The gels were not "insoluble" like formalin induced gels, and they could be diluted with warm water to produce apparently normal solutions. They could not, however, be pipetted so the results obtained on gelatine 877/1 on day 10 that were obtained by taking 7.2 g and 2.0 g of sample and diluting them appropriately for colour and fluorescence determinations, must be considered suspect.

Ribose gelatine interaction - Induced colour.

The development of colour due to the Maillard reaction was not the same for the two different gelatines. The gelatines chosen for this study were normal gelatines of similar colour and from the start of extraction. The difference in Maillard reaction colour development must be primarily a function of differences in the availability of amine groups for the reaction. As amine groups were involved in all known cross-linking reactions, for example, Kuypers, Tyler, Kurth and Horgan (1994), it was of interest to note that not all reactions at the lysine side chains led to colour development. Conversely it was concluded that differences in the cross-linking of collagen could limit the development of colour due to the Maillard reaction.



The effects of gelatine ribose interaction on (C) colour, (F) Fluorescence intensity and (P) solution pH, at starting — pH 9 & - - pH 6. Gelatines 155/1 & 877/1 had similar gel strength and colour.

Figure 14. Gelatine ribose interaction. (10% gelatine w/w in 0.2M aqueous ribose solution, incubated at 37°C).

It was also noteworthy that although colour development during days 1 to 4 was faster at pH 9 than at pH 6, eventually the pH 6 solution colour caught up with and outstripped the pH 9 colour which solved the apparent anomaly observed in the "Preliminary Study No 1" above. Furthermore, the development of colour in Figure 14 showed a distinct "lag phase" at pH 6 which was hardly visible at pH 9, particularily with gelatine 155/1.

It was noted that the absorbance spectra of the ribose treated gelatines exhibited an anomaly at about 470 nm when compared to normal gelatine absorbance spectra. This resulted in the ribose containing reaction mixtures having a distinctly reddish tinge when compared to normal or glucose treated gelatine as shown in Figure 15 below. Towards the end of the reaction time however, the solutions became too dark for the reddishness to be evident.

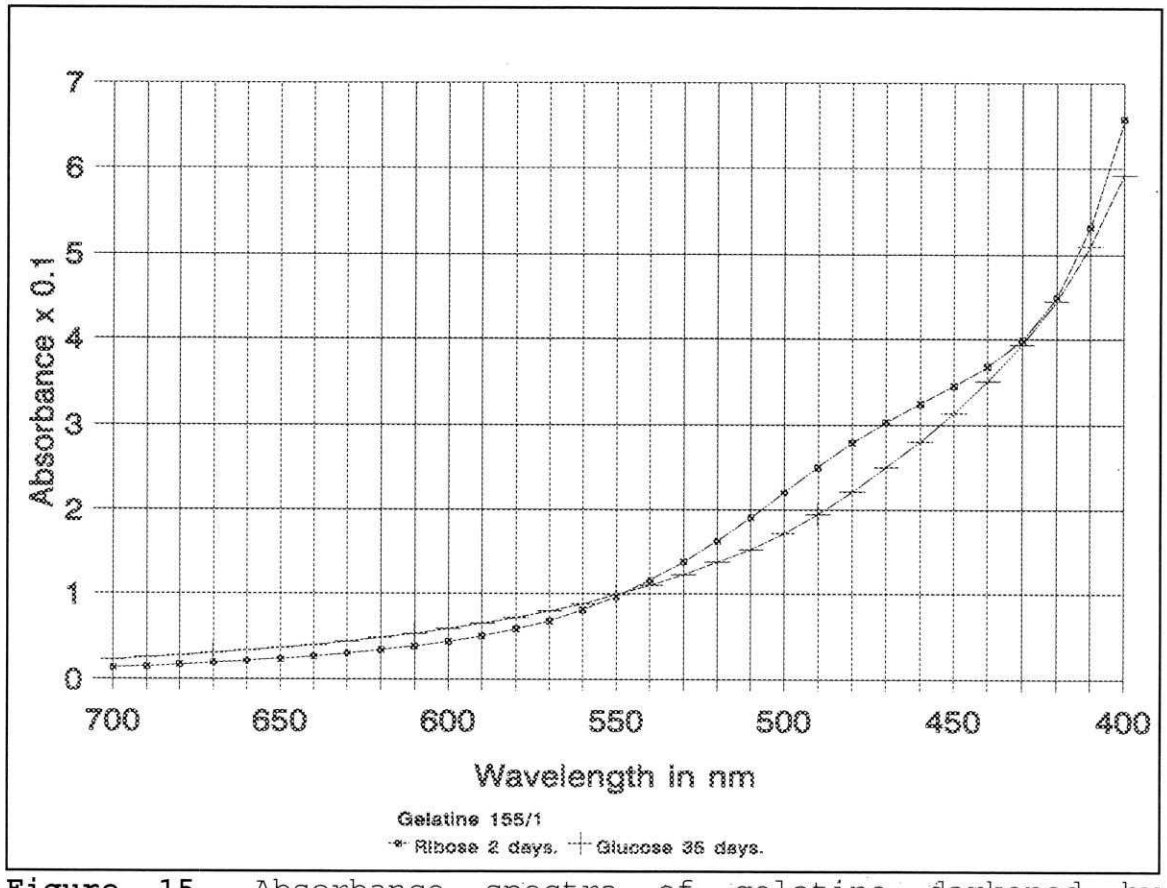


Figure 15. Absorbance spectra of gelatine darkened by incubating 10% w/w gelatine in 0.2 M glucose or ribose aqueous solution at 50°C or 37°C respectively.

Finally, from a comparison of the slopes of the glucose and ribose induced absorbance areas against time for gelatines 155/1 and 877/1 at pH 6:

Glucose averaged 1.94 absorbance units per day for 84 days at 50°C.
Ribose average 21.3 absorbance units per day for 11 days at 37°C.

Hence, it was concluded that the reaction rate of gelatine with ribose at 37°C was some 10.9 times faster than the reaction between gelatine and glucose at 50°C. If one then accepts that the reaction rate doubles for every 10°C (Findlay, 1947) then the ribose reaction rate was some 28 times faster than the glucose reaction rate at the same temperature which was in reasonable agreement with the factor of 25 given by Tanaka *et al*. (1989).

Ribose gelatine interaction - Induced fluorescence.

As with colour, it was evident (Figure 14) that both gelatines had not responded identically in their reaction with ribose. Again gelatine 155/1 reacted faster than 877/1 reaching the maximum fluorescence at pH 6 in about 7 days as against 9 days for 877/1.

The downturn in the fluorescence intensity plots was again an example of the phenomenon of concentration quenching because the fluorescence intensities of the pH 9 solutions diluted to 0.01% (0.1 ml to 10 ml water) showed a continuous increase to a maximum intensity and then a levelling off as gelation occurred. (See detailed results in APPENDIX 5. RB-FI/100 and RD-FI/100).

The fluorescence spectra were studied in detail from time to time. Although efforts were made to detect the 370 nm excitation maximum with emission at 445 nm which Tanaka *et al*. (1988) found with ribose reaction on collagen, no sign of these fluorescence peaks could be found. The excitation spectrum shown in Figure 16 shows a single maximum at ±345 nm (due to monitoring the emission at 420 nm) but no sign of a further maximum near 370 nm. On all occasions the excitation maximum was close to 335 nm and the emission spectrum showed an inflection at 385 nm. This was consistent with the generation of the pentosidine cross-link, and a continuously increasing emission to about 410 nm as shown in Figure 17.

Ribose gelatine interaction - Induced pH changes.

It was evident from Figure 14 that there was an approximately linear drop in the pH of the reaction mixtures with time. This would be consistent with the removal of amino groups due to the Maillard reaction. The only anomaly was the pH of the gelatine 877/1, pH9 reaction mixture after 10 days. Perhaps as gelation occurred the Maillard reaction slowed markedly or ceaseed. This also seemed to be the case with colour development because the increase in colour production of this mixture also appeared to cease after gelation, although no measurements could be made to substantiate this observation.

Ribose gelatine interaction - Controls.

From ADDENDUM 5. it could be seen that for the reaction mixtures without ribose RE, RF, RG, RH, there was initially a small increase in colour and fluorescence and a decrease in pH over the first few days of incubation, whereafter the values remained substantially constant. This would be consistent with the completion of any incomplete Maillard reaction inherent in the gelatine. Against this, however, it must be stated that the gelatines were the product of long liming, that is, the raw material had been immersed in lime at greater than pH 11 for at least 30 days, hence, it was hard to envisage how there could be any sugars present that had not completely reacted with the collagen prior to its conversion to gelatine, unless, as indicated above, the Maillard reaction becomes particularly slow in the solid or gel state due to steric hindrance and the inability of the reactants to come together in the required conformation. It was also possible that sulphide in the lime liquor could have been responsible for inhibiting the Maillard reaction during conditioning, but the failure to observe an effect on colour due to sulphide would probably discount this possibility.

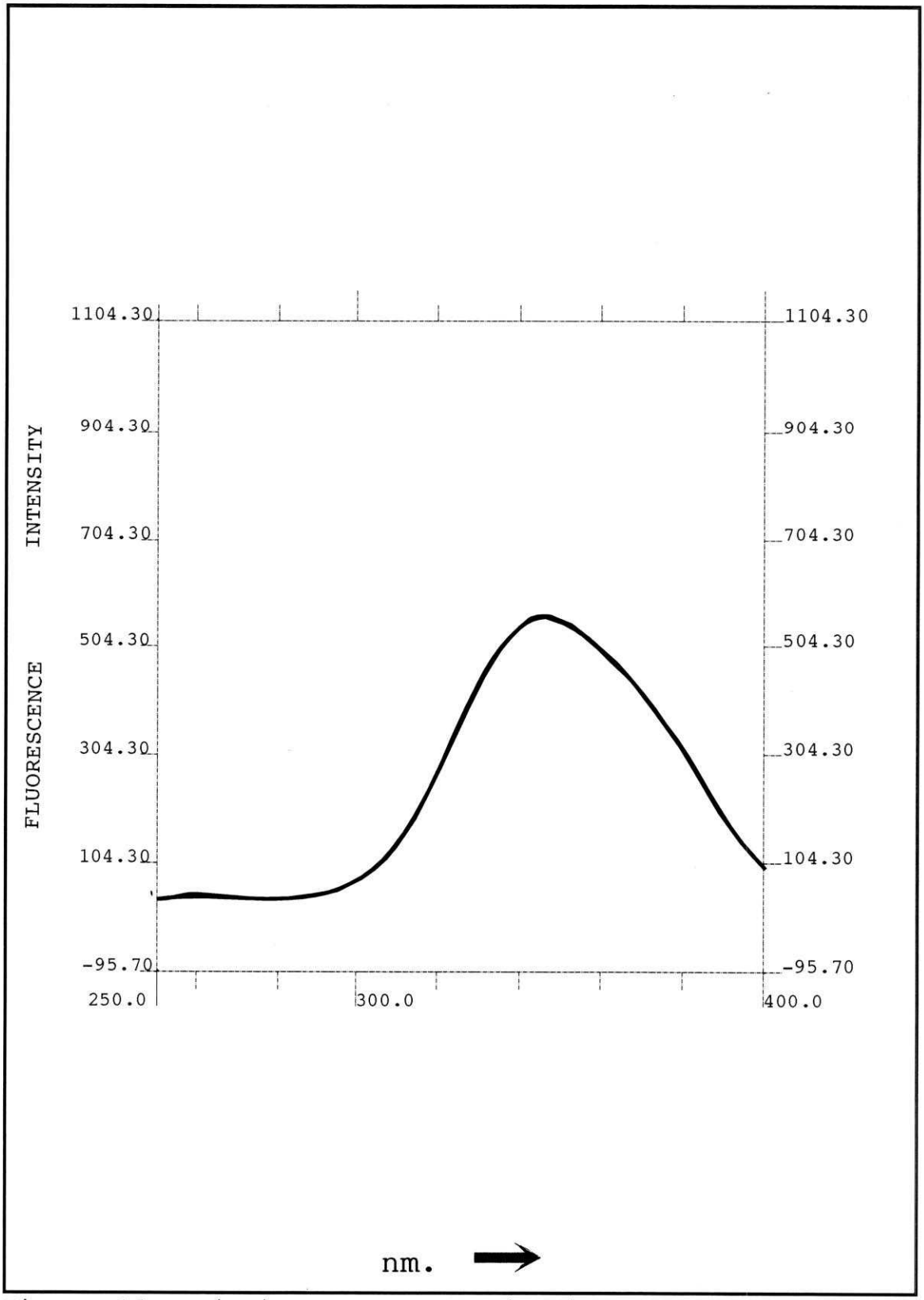


Figure 16. Emission 420 nm. Excitation spectrum of ribose darkened gelatine (155/1). A 10% w/w solution of gelatine in 0.2 M ribose at an initial pH of 6.0 incubated at 37°C for 7 days.

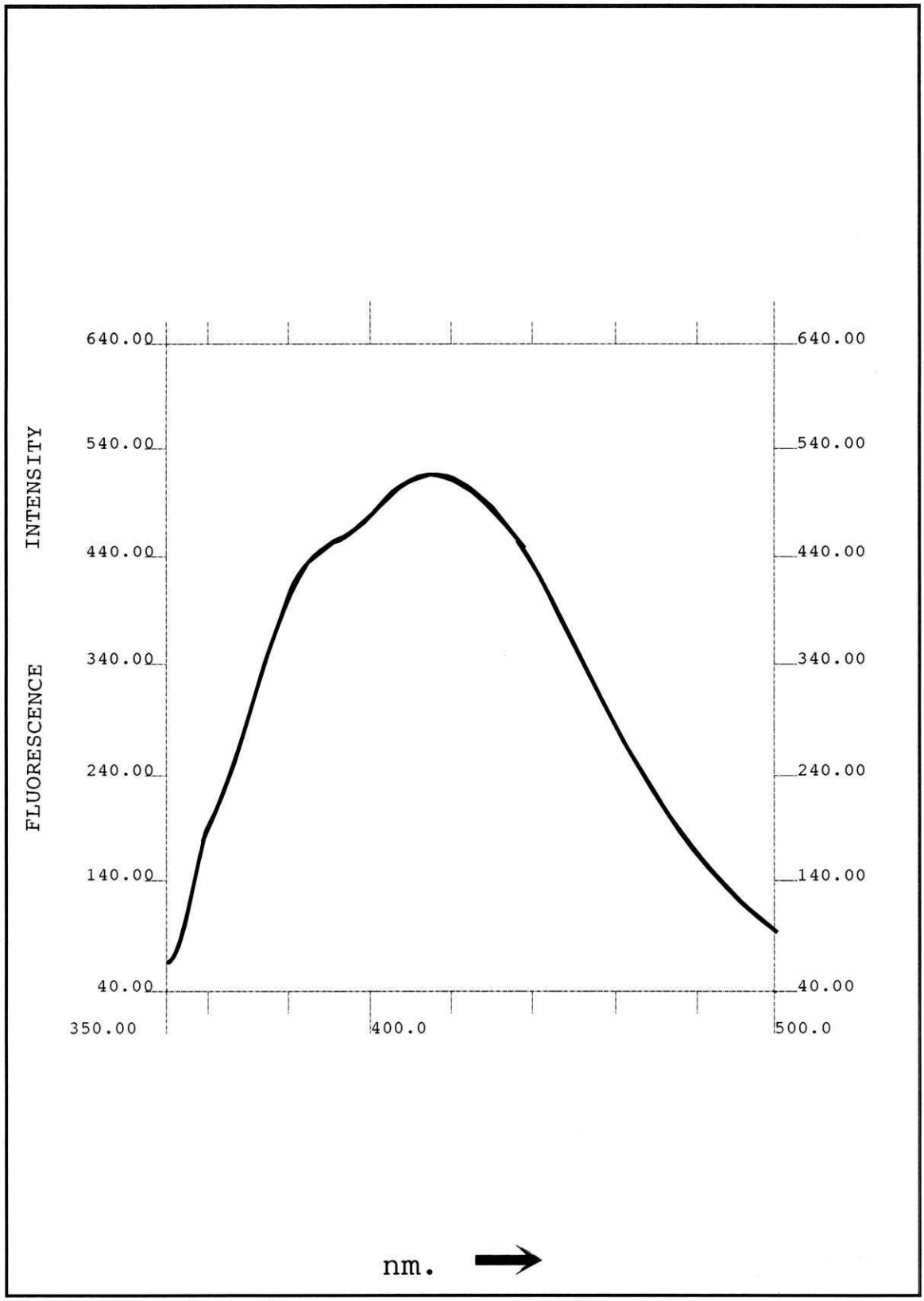


Figure 17. The emission spectrum of ribose darkened gelatine (155/1) at 1% concentration. Gelatine 10% w/w in 0.2 M ribose solution, after 7 days at 37°C with a starting pH of 6.0. Excitation at 335 nm. Note the shoulder at ca. 385 nm and the peak at 410 nm.

PENTOSIDINE SYNTHESIS.

In the study of Sell and Monnier (1989) "pentosidine" was synthesised by heating 100 mM quantities of L-arginine, L-lysine and D-ribose in 3 litres of water for 1 hr at 80°C (pH 7.3) and then purifying the product by ion exchange and chromatography. They showed that the purified product was identical to the natural "pentosidine" from human dura mater, however, it was clear from the results that pentosidine was not the only product of reaction. Hence, it was decided to repeat the Sell and Monnier synthesis under conditions more similar to those employed in the gelatine aldose reactions above. The reaction mixture consisted of equal amounts of 0.02M lysine.HCl, arginine.HCl, and ribose in 0.02M ammonia. The solution had a pH of 9.5.

Table 8. *Fluorescence development in an equimolar mixture of lysine, arginine and ribose at room temperature (± 20°C) and at 37°C.*

Fluorescence Intensity due to Excitation at 335 nm.				
Emission at	385 nm.		405 nm.	
Time.	Reaction Temperature.		Reaction Temperature.	
	Ambient.	37°C	Ambient.	37°C
Zero	5.0			
10 min.	5.0			
3 days.	15	100		
10 days.	135	982	156	797
10 days. Diluted 1/100		103		88

The conclusions drawn from Table 8. were:

1. The formation of fluorescent products was greatly accelerated by the increase in temperature from ambient (20°C) to 37°C.

2. The 10 day fluorescence exhibited marked concentration quenching as shown by a 100 times dilution exhibiting only a 10 times reduction in fluorescence intensity.

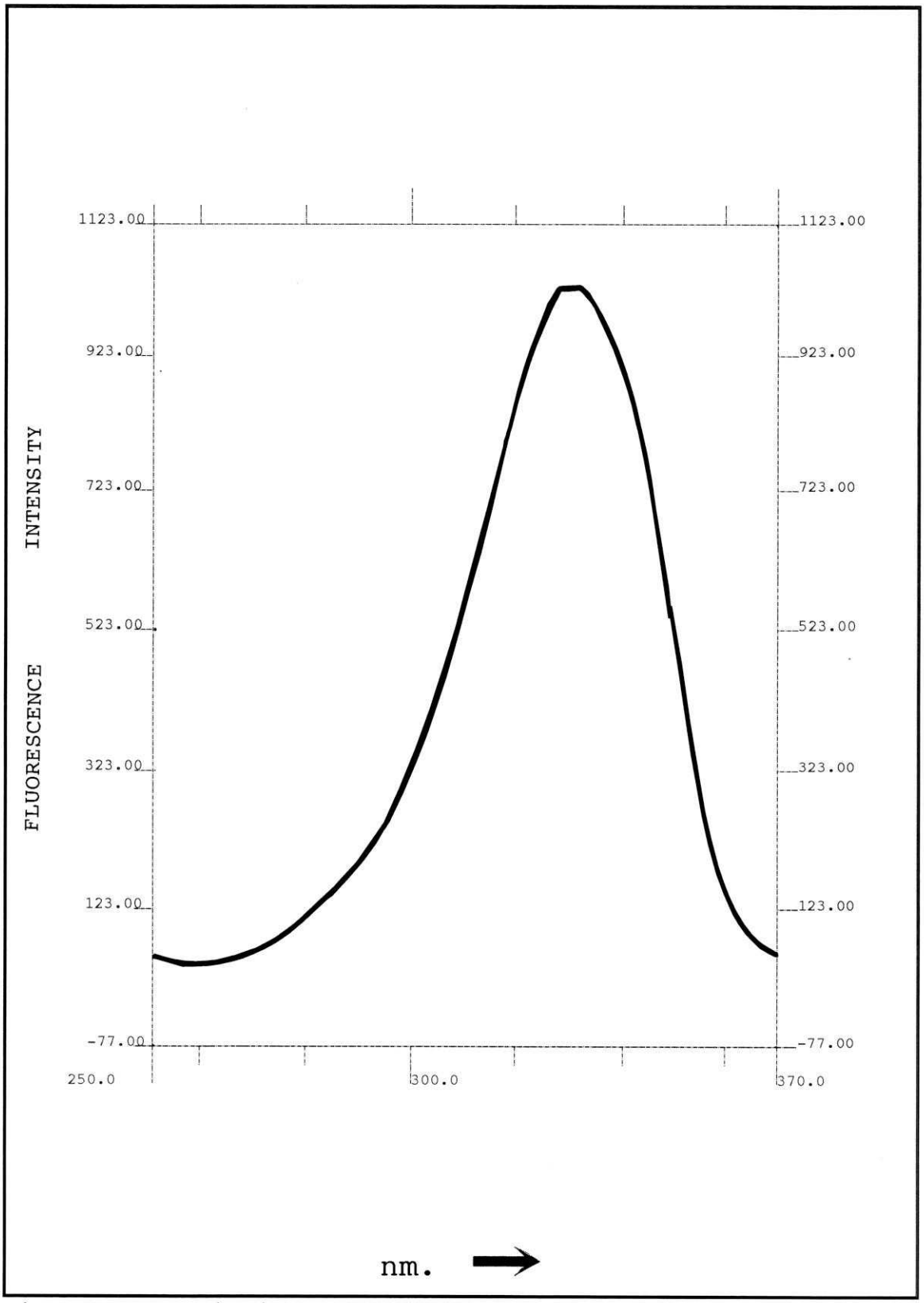


Figure 18. Emission at 385 nm. Excitation spectrum of the "pentosidine" reaction mixture after 10 days at 37°C. Note the 335 nm excitation peak. (Reaction mixture = L-lysine, L-arginine and D-ribose each at 0.02 M concentration and pH 9).

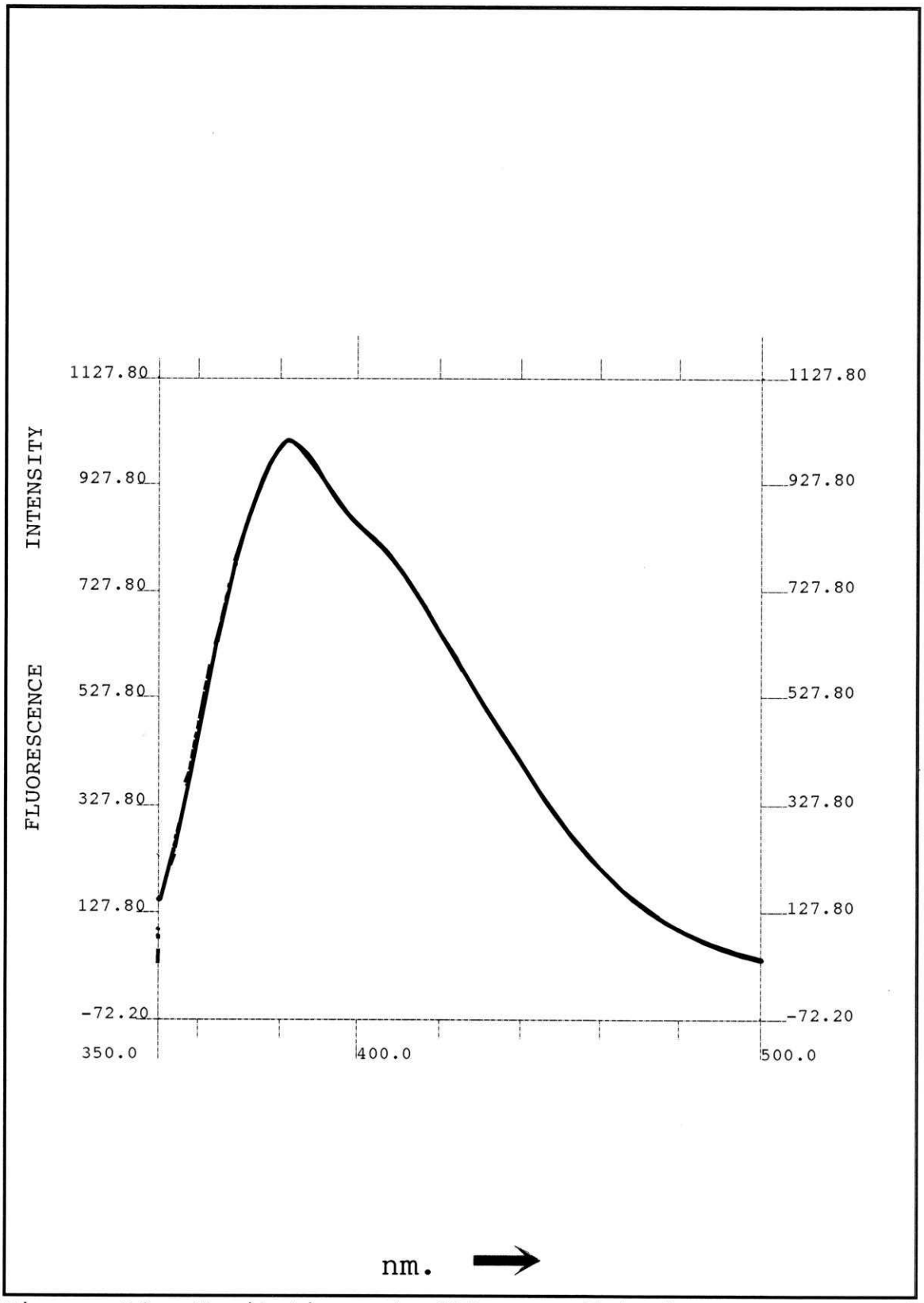


Figure 19. Excitation at 335 nm. Emission spectrum of "pentosidine" reaction mixture after 10 days at 37°C. Note the 385 nm emission maximum and the shoulder at ± 410 nm. (Reaction mixture = L-lysine, L-arginine and D-ribose each at 0.02 M concentration and pH 9)

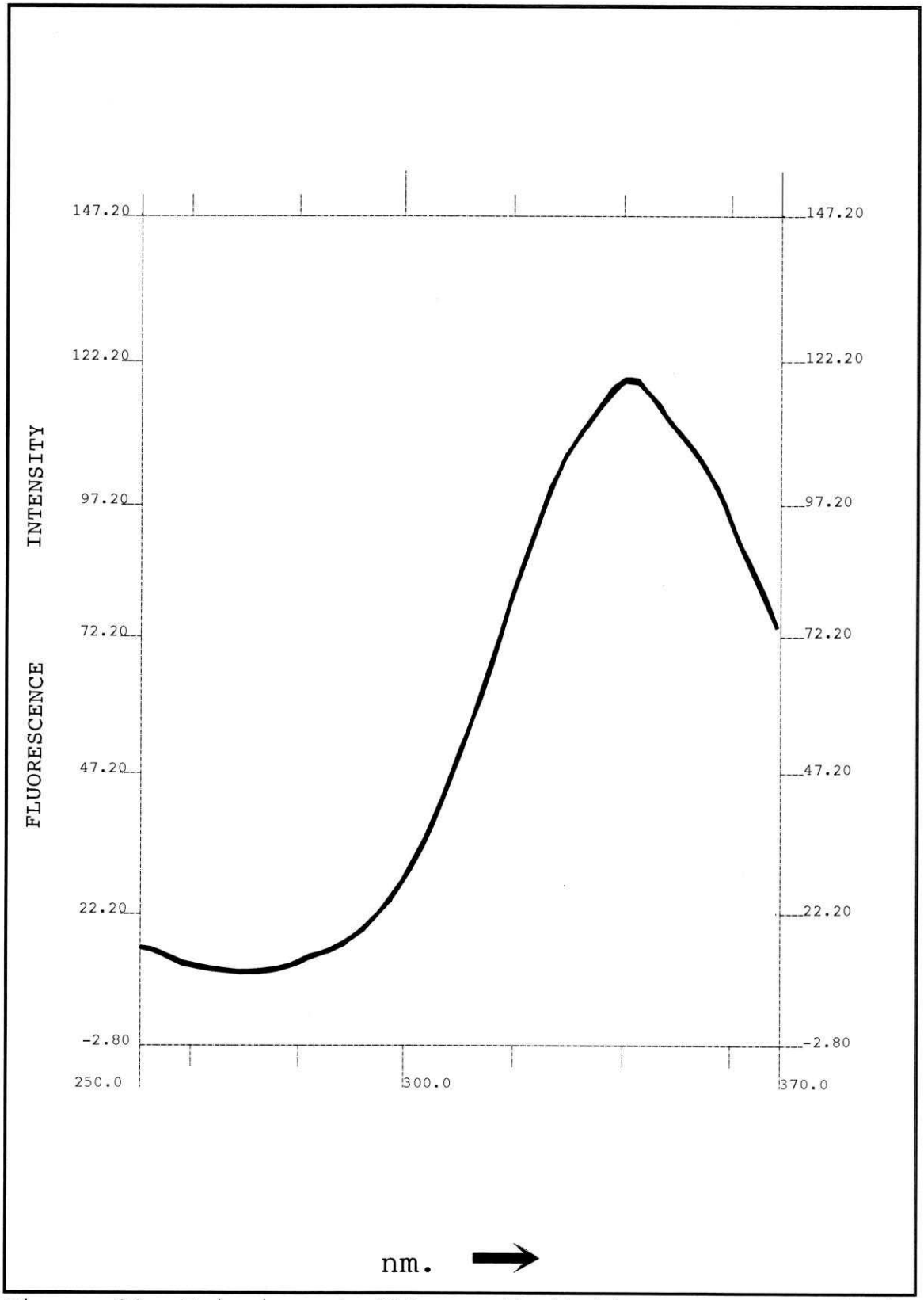


Figure 20. Emission at 385 nm. Excitation spectrum of the "pentosidine" reaction mixture after 10 days at ambient temperature (20°C). Note the 340 nm excitation maximum. (Reaction mixture = L-lysine, L-arginine and D-ribose each at 0.02 M concentration and pH 9).

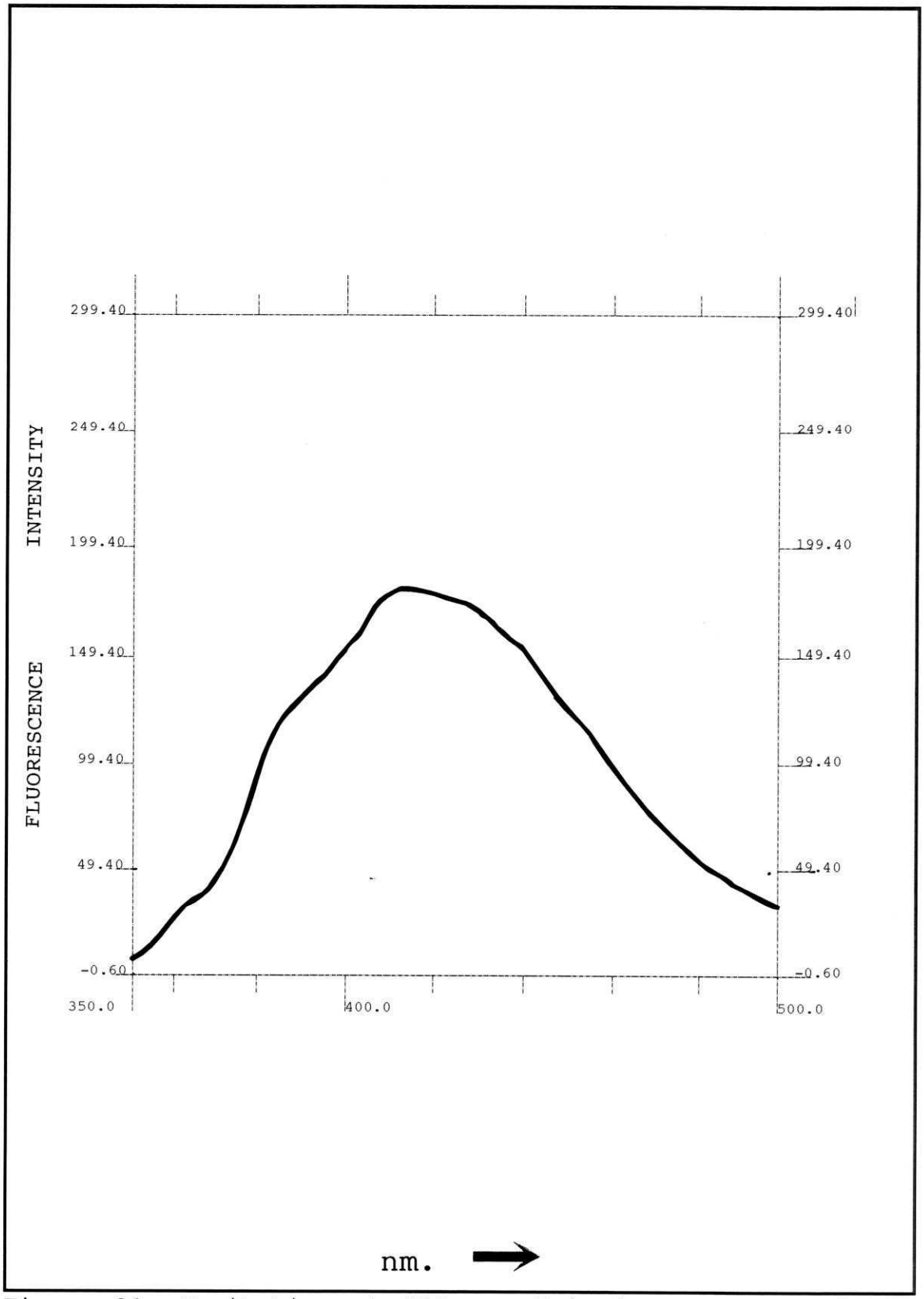


Figure 21. Excitation at 335 nm. Emission spectrum of the "pentosidine" reaction mixture after 10 days at ambient temperature (20°C). Note the 410 nm emission maximum. (Reaction mixture = L-lysine, L-arginine and D-ribose each at 0.02 M concentration and pH 9)

3. As with colour formation the pentosidine reaction appeared to have had an initiation phase followed by a much faster reaction phase which was typical of free radical catalysed chain reactions such as the decomposition of acetaldehyde (Glasstone, 1952).

From Figures 18 to 21 it was observed that fluorescence spectra similar to those of gelatine (Figure 5) were produced from the slow reaction of lysine, arginine and ribose at ambient temperature. That is, fluorescence with excitation at 335 nm and emission from 385 to 410 nm was produced at lower temperature while at 37°C the emission intensity peaked at 385 nm and fell away rapidly at higher wavelengths. This latter was characteristic of the production of pentosidine.

THE EFFECT OF ANION EXCHANGE.

As can be seen from Addendum 2 and Table 9 the effect of anion exchange on the colour of gelatines 939/4 and 972/6 was marked. This phenomenon had been found to be most obvious in the case of dark, low quality (Bloom strength and viscosity) gelatines.

Table 9. *The spectrophotometric data on two gelatines before and after anion exchange.*

	Visual Colour	Estimated Colour*	Absorbance# 400 nm.	Absorbance# 700 nm.	Absorbance# Area 400-700 nm.
Sample	Before Anion Exchange				
939/4	14.5	15.6	0.366	0.017	22.37
972/6	17.5	16.4	0.369	0.017	23.46
178/12 Control	17.8	15.7	0.366	0.017	22.37
Sample	After Anion Exchange				
K939	9.4	10.3	0.232	0.009	14.71
Q939	9.2	10.1	0.229	0.010	14.4
F972	11.7	11.9	0.266	0.013	16.79

Solvent = ES1. Concentration = 6.67%. Filter = 0.45μ membrane.
* Absorbance Area x 0.7

Table 9 showed that the bleaching effect due to anion exchange was variable

and in terms of reduction in absorbance was about 37% for gelatine 939/4 and only 27% for gelatine 972/6. Table 9 also showed that anion exchange resulted in colour reductions of 5 to 6 Davis units and from Table 10 it was clear that there was virtually no change in the 335/385 nm fluorescence intensities of the gelatines as a result of anion exchange. From the fluorescence/colour correlation determined earlier, the fluorescence data of K939 and Q939 indicated that these gelatines should have had colours of about 14.6 and the F972 gelatine a colour of 15.9 which was correct for the original gelatines but not for the anion exchanged ones. This result clearly showed that there was a component of the colour of gelatine that was not related to fluorescence. This finding helped to account for the relatively poor correlation coefficient between fluorescence and colour. The fact that this component had been found in dark, low quality, gelatines also helped to explain why the correlation between colour and fluorescence was not significant for third extraction gelatines as noted in the discussion on Table 2.

Table 10. *Fluorescence Intensity data for 1% w/v solutions of two gelatines before and after anion exchange.*

FLUORESCENCE INTENSITY		
Excitation Wavelength. Emission Wavelength.	335 385	370 440
Sample.	Before Anion Exchange.	
939/4	253	141
972/6	274	149
178/12 Control.	288	177
Sample.	After Anion Exchange.	
K939	269	128
Q939	254	129
F972	272	137

The very dark regenerant solution from regeneration of the column after it had treated about 1200 bed volumes of 3% w/v gelatine liquor or 15.7 kg of gelatine, had a pH of 12.6, and was only about 0.07 N NaOH after it had regenerated the resin. Normally fluorescence determinations were done on 1% w/v solutions of gelatine. The fluorescence determination on the alkaline

regenerant solution was equivalent to determining the fluorescence of a 31% solution of gelatine. The determination was also done on the acidified regenerant because Sell and Monnier (1989) had found that the fluorescence of pentosidine was completely quenched at pH 9. Hence due to dilution the fluorescence of the acidified regenerant should have been about 59% that of the alkaline regenerant solution if no "quenching" was involved. The 335/385 fluorescence intensities of 87 for the alkaline regenerant and 102 for the acid regenerant indicated that pH quenching was a factor, however the low values were taken to indicate that little of the colour removed by anion exchange was associated with pentosidine or Maillard fluorescence.

Anion exchange of ribose darkened gelatine.

In order to ascertain that the 100 ml column was bleaching a dark gelatine normally the column was used to treat solutions of gelatine 178/12, which had acted as a "control" in the colour determination study, with the results shown in Table 11.

Table 11. *The effect of anion exchange on gelatine 178/12.*

Absorbance using Filter No. 2.		
Before Anion Exchange Treatment	After Anion Exchange Treatment	Decrease
0.134	0.102	0.032 (24%)
0.136	0.104	0.032 (24%)

The pale gelatine 877/1 that had been used extensively in the glucose and ribose interaction experiments was chosen for the following investigation involving ribose darkening because it was known that the gelatine exhibited minimal bleaching due to anion exchange. Hence, any observed bleaching after ribose darkening could not be attributed to similar bleaching prior to ribose treatment. Furthermore, controls without ribose were included in the experiment.

Table 12. *The effect of anion exchange on ribose darkened gelatine 877/1.*

Incubation Days.	Absorbance using Filter No. 2.			
	Sample with Ribose	Before Anion Exchange.	After Anion Exchange.	Decrease due to Anion Exchange.
6	Excluded	0.048	0.037	0.011 (23%)
	Included	0.255	0.229	0.026 (10%)
7	Excluded	0.054	0.040	0.014 (26%)
	Included	0.310	0.264	0.046 (15%)

Table 11 showed that gelatine 178/12 lost 24% of its absorbance due to anion exchange which was of the same order of magnitude of the reduction obtained with gelatines 939/4 and 972/6 in Table 9. From Table 12 it was concluded that ribose darkened gelatine after 6 and 7 days incubation lost only 10% to 15% of its colour after anion exchange which was considerably less than the anion exchange bleaching of normally dark gelatines. Hence it seems probable that gelatine colour produced by the Maillard reaction was only slightly susceptible, if at all, to bleaching or absorption using anion exchange resin.

CONCLUSIONS.

1. The observation that gelatine exhibited 335/385 "pentosidine" fluorescence was evident from Table 1, however, most gelatines also had a second peak in the emission spectrum at about 410 nm. This pair of peaks was also produced by the Sell and Monnier (1989) pentosidine synthesis mixture when it was held at ambient temperature for 10 days. Hence, it could be proposed that both the fluorescence peaks found in gelatine were the products of the Maillard reaction with slightly different molecular structures.

In the case of pigskin gelatines, the second emission peak due to 335 nm excitation was closer to 420 nm, as was the case with fishskin gelatines. The small change from 410 to 420 nm could possibly be a function of the difference in processing (the presence or absence of alkaline conditioning).

2. Tanaka *et al*. (1988) produced fluorescence and cross-linking in collagen by reacting it with ribose and glucose. This experiment was repeated with gelatine in place of collagen and it was found that the darkening of the solutions with time was accompanied by an increase in 335/385 fluorescence intensity. In the case of ribose, the reaction mixtures showed a marked lag phase at pH6. Also these mixtures gelled at 37°C which could not be a clearer demonstration that the Maillard reaction caused cross-linking. The increase in 335/385 fluorescence was accepted as showing that the cross-linking was due to formation of pentosidine.

3. The absorption spectrum of ribose cross-linked gelatine was quite different from the spectrum of the glucose cross-linked gelatine. The absorption spectrum of glucose cross-linked gelatine and the spectrum of naturally dark gelatine were virtually identical and this was interpreted as indicating that the natural pentosidine cross-links in collagen and gelatine were derived from glucose rather than ribose *in vivo*. This confirmed the contention of Dyer *et al*. (1991) that pentosidine was formed from glucose with the loss of one carbon atom, rather than from ribose. Hence, the scarcity of ribose to take part in cross-linking, as mentioned by Sell and Monnier (1989), was not relevant.

4. In the case of glucose, the rate of reaction with gelatine was about 28 times slower than with ribose. This had implications in production where even with raised temperatures, as in evaporation or sterilization, the amount of the Maillard darkening should be expected to be small to negligible due to the short times involved. The low reaction rate also explains how confectionery manufacturers could process glucose and gelatine at high temperatures without any apparent darkening.

5. There was a significant correlation between overall gelatine colour and animal age and between gelatine colour and 335/385 nm fluorescence. Based on the findings of Uchiyama *et al*. (1991), that 335/385 nm fluorescence was due only to pentosidine, it was concluded that the colour of gelatine was a function of the formation of the Maillard pentosidine cross-links. Furthermore, there was a significant inverse correlation between gelatine colour and hide extractability. This was additional evidence of the formation of stable cross-links (including pentosidine) in collagen, with senescence.

It could be argued that only the isolation of pentosidine from gelatine would

unequivocally prove that the colour of gelatine was caused by the pentosidine cross-link however Odetti *et al.* (1994) also based their conclusions on fluorescence data only, hence, fluorescence data alone was accepted as sufficient for meaningful conclusions to be drawn.

6. The significant correlation between first and second extraction high quality gelatine colour and fluorescence but the failure of the correlation with low quality gelatine was explained by the finding that the bleaching action of anion exchange resin had virtually no effect on the fluorescence of the gelatine. This was interpreted as showing that there were at least two sources of colour in gelatine; the first was the Maillard reaction which was not susceptible to anion exchange bleaching and the second was an as yet unknown source of colour which was susceptible to anion exchange bleaching.

7. Tanaka *et al.* (1988) noted the development of 370/440 nm fluorescence with the ribose treatment of collagen. This fluorescence was sought throughout this study of gelatine, and it was only found with Type A gelatine from calf skin, that is, from animals of less than 6 months of age. This fluorescence was not found with Type A gelatines from pigskin nor was it found in Type B gelatine from 10 month old animal skin. There was insufficient information on which to base theories as to the origins of the fluorescence other than to suggest it could be associated with immature collagen or that it was caused by a very unstable or short-lived cross-link.

8. The absence of 295/395 nm pyridinoline fluorescence in Type B gelatines was expected (Eyre, 1988) hence finding it in Type A calf skin even if only at a low intensity indicated that the pyridinium cross-link was present in bovine hide collagen and this possibly explained the conditioning phenomena encountered in this study:

(i) Eyre, Paz and Gallop (1984) stated that the pyridinium cross-link was labile in alkali.

(ii) The amount of pyridinium cross-link in collagen was invariant. (Uchiyama *et al.* 1991).

(iii) This study showed that young animal hide extractability increased steadily with conditioning time, but old animal hide exhibited a limit to the extractability after which conditioning time seemed to have had no effect.

Hence, if bovine hide contained a small amount of the alkali labile pyridinium

cross-link which predominated in younger animals and which was later swamped by pentosidine and other mature cross-links as the animal aged, then it would be expected that the time related response to alkali conditioning in younger animals would decrease as the animal became older and as the alkali labile cross-links became relatively fewer.

9. The ribose interaction with gelatine at pH 8 was of particular interest because it indicated that after gelation the pH and colour formation slowed dramatically indicating that steric hindrance may play an important role in the rate of the Maillard reaction *in vivo*. Current treatments to control the effects of high glucose levels in diabetes were based on making amine groups available for reaction with excess glucose, hence the patient could be medicated with amino acids (Ulrich and Cerami, 1992). Possibly some means of using steric hindrance to limit the effects of glucose in the body could be considered. Likewise this study highlighted the effect of pH on the rate of the Maillard reaction and perhaps this effect could also be applied medicinally.

The sugar/gelatine interaction experiments also indicated that two similar gelatines reacted with the glucose or ribose at very different rates. It could only be surmised that this effect indicated different availabilities of amine groups for reaction but the as yet unknown reason for this difference in availability could be significant when it came to differences in rates of senescence.

10. The controls for the glucose/gelatine interaction experiments indicated only a small amount of darkening of gelatine solutions due to storage at pHs of 6 and 8.75 at 50°C. for many weeks. The observation that the darkening was greater at pH 6 than at pH 9 for both gelatines could indicate that the chromophore was not related to the Maillard reaction. This was confirmed by the pH 6 fluorescence (335/385 nm) being less than or equal to the pH 9 fluorescence for both gelatines. (Addendum 4). The ribose/gelatine interaction controls were held at 37°C for only 14 days which was not long enough for material discolouration etc. to occur.

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A D D E N D A

ADDENDUM 1. Origins of the gelatines used in this study.

Laboratory Gelatines:

YS	10 month old Afrikaner - conditioned with lime sulphide.
CT	18 month old Brahman - conditioned with lime & sulphide
CTO	12 yr. old Afrikaner - " " "
5Y	5 yr. old Afrikaner - " " "
ST	12 yr. old Afrikaner - " " "
IOB	12 yr. old Inguni - " " "
6Y	6 yr. old Friesland - " " "
WT	12 yr. old Afrikaner - " " "
3Y	3 yr. old Friesland - " " "
C	Gelatine from chrome tanned leather.
FISH	Acid process gelatine from fish skin.
D	Acid process pigskin gelatines.

For complete processing and quality details see the Chapter 2 on the origins of gelatine colour.

155/1 First extraction gelatine.
877/1 First extraction gelatine.
(Both gelatines were chosen for their normal but pale colour.)

143/1 First extraction gelatine.
23/2L Gelatine of medium colour.
939/4 Dark gelatine.
K939 & Q939 Gelatine recovered from an anion exchange column after treatment of solutions of gelatine 939/4.
972/6 Dark gelatine.
F972. Gelatine recovered from an anion exchange column after treatment of a solution of gelatine 972/6.

ADDENDUM 2. Analysis Data of Gelatines.

Gelatine	155/1	877/1	23/2L	143/1
Bloom	269	267	241	216
Colour	6.8	5.6	8.6	12.3
Clarity	12.5	11.5	12.5	10.5
pH 1%	5.7	5.1	5.3	5.2
Moisture %	10.4	10.6	10.8	9.5
Ash %	0.4	0.4	0.23	0.65
Viscosity ms.	36	42	33.9	32.5

Gelatine	939/4	K939	Q939	972/6	F972
Bloom	170	140	159	75	74
Colour	14.5	9.4	9.2	17.5	11.7
Clarity	11.5	11.0	12.0	11.0	10.5
pH 1%	5.6	6.0	5.8	5.5	5.5
Moisture %	7.9	9.8	10.9	10.7	9.4
Ash %	1.6	2.2	1.8	1.7	2.3
Viscosity ms.	27.8	19.2	24.1	19.7	17.7

ADDENDUM 3. Fluorescence data.

EXCITATION EMISSION		335 nm 385 nm	295 nm 380 nm	370 nm 440 nm	335 nm 420 nm
SAMPLE CODE	COLOUR	Peak Ht.	Peak Ht.	Peak Ht.	Peak Ht.
YSA/1	3.6	41	32		
YSA/2	4.4	50	34		
YSA/3	4.8	75	43		
3Y4A/1	6.6	102	73		
CT6/1	5.2	115	83		
5Y6A/1	6.4	118	73		
5Y6A/2	4.4	134	86		
5Y4/2	6.8	135	77		
3Y4A/2	6	138	70		
CT6/2	5.6	144	89		
CT6/3	6	148	88		
5Y4/3	8.4	151	86		
5Y6A/3	9.4	158	90		
6Y4B/2	8	164	114		
IOB/1	16	169	112		
6Y4B/3	9.5	174	106		
6Y4B/1	6.4	183	104		
IOB/2	16	207	112		
IOB/3	20	254	127		
5Y4/1	5.6	104			
5Y4/2	6.8	126			
5Y4/3	8.4	149			
5Y6/1	6.4	115			
5Y6/2	8.4	128			
5Y6/3	9.4	148			
ST16/1	10.7	174			
ST16/2	12.3	192			
ST16/3	14.5	228			
178/12	17.8	261			
ST12/1	6.4	105			
ST12/2	10	124			
ST12/3	10.7	167			
INOE/1	9.4	175			
INOE/2	12.3	207			
850/6	17.8	249			
850/6C	17.8	250			
C542/1L	1.6	63			
C542/2L	1.9	64			
5Y4/1	5.6	94			
5Y4/2	6.8	129			
5Y4/3	8.4	142			
6Y4A/1	8.4	141			
6Y4A/2	7.6	165			
6Y4A/3	8.6	174			
YSA/1	3.6	38			
YSA/2	4.4	48			

ADDENDUM 3. Continued.

EXCITATION EMISSION		335 nm 385 nm	295 nm 380 nm	370 nm 440 nm	335 nm 420 nm
SAMPLE CODE	COLOUR	Peak Ht.	Peak Ht.	Peak Ht.	Peak Ht.
YSA/3	4.8	68			
ST24/1	8.9	133			
ST24/2	12.3	168			
ST24/3	13.3	214			
WT4/1	11.4	117			
WT4/2	12.3	130			
WT4/3	13.3	172			
CT4/1	5.6	104			
CT4/2	5.6	133			
CT4/3	5.6	142			
CT04/1	10	187			
CT04/2	9.4	223			
CT04/3	10	239			
CALF TyA/1	4	21		121	
CALF TyA/2	3.6	27		183	
CALF TyA/3	5.2	49		211	
155/1	6.8	147		86	
877/1	5.6	162		90	
D31108	3.8	127			245
D2227	2.2	112			182
D2116	2	87			140
D1512	2.2	78			142
D2249	2.4	104			153
D147	2.4	81			135
D32117	4.7	156			267
D1278	2.2	85			136
D3264	4	122			212
FISH-COD2/1	6	50			67
FISH-824/1	11.4	51			72
C200/1	<3.2	61			

385 nm Fluorescence/ Colour - Regression Output:

Constant	0.006579	
Std Err of Y Est	2.677614	
R Squared	0.60754	R = 0.78
No. of Observations	75	
Degrees of Freedom	73	
X Coefficient(s)	0.057929	
Std Err of Coef.	0.005449	

ADDENDUM 4. Glucose gelatine interaction.

GELATINE + GLUCOSE
AREA UNDER THE ABSORBANCE CURVE 400 TO 700 nm. (Colour)

SAMPLE	A	B	C	D
Gelatine	155	155	877	877
	pH-6	pH-9	pH-6	pH-9
Day No.				
0	7.8	10.3	9.3	10.7
7	15.5	28.8	12.5	19.9
14	18.2	61.3	14.2	48.3
21	23.5	90.7	22.9	71.2
28				
35	40.6	126.2	35.3	102.3
42	49.8	132.8	45.6	116.9
49	58.1	97.1	56.8	75.4
56	94	165	63.5	86.7
63	114.9	241.5	126.8	140.6
70	147.4	272.6	144.4	171.3
77	160.2	299.6	163.7	204.6
84	185.2	338.7	157.8	238.3

GELATINE + GLUCOSE
FLUORESCENCE INTENSITY.

SAMPLE	A	B	C	D	*B/100	D/100
Gelatine	155	155	877	877		
	pH-6	pH-9	pH-6	pH-9		
Day No.						
0	151	154	157	155		
7	160	535	176	308		
14	200	617	212	562		
21	210	577	280	540	23	16
28						
35	272	650	320	701	31	34?
42	282	602	313	503		
49	331	>1000	368	458		
56	402	585	376	396	11?	
63	402	362	394	351	41	14
70	363	252	353	310		
77	343	248	350	309	33?	21
84	330	211	373	282	43	21

*Samples B and D diluted 1/100.

ADDENDUM 4. Continued...

GELATINE + GLUCOSE
1% SOLUTION pH

SAMPLE Day No	A	B	C	D
0	-	-	-	-
7	-	-	-	-
14	5.36	7.62	5.99	7.49
21	5.39	7.2	6.1	7.18
28	-	-	-	-
35	5.35	6.62	5.8	6.35
42	5.4	6.59	5.72	6.75
49	5.29	5.4	5.64	5.52
56	5.37	6.27	5.65	5.56
63	5.42	6.11	5.54	5.42
70	5.38	6.15	5.62	5.39
77	5.46	6.19	5.5	-
84	5.32	6.05	5.49	5.18

- Values not determined

C O N T R O L S

DAILY INSTRUMENT CONTROLS.*

Gelatine. Day No.	COLOUR AREA 155	COLOUR AREA 877	FLUORESCENCE 155	FLUORESCENCE 877	pH 155	pH 877
0	9.72	7.79	152	156	-	-
7	10.57	8.7	140	147	-	-
14	10.46	8.09	147	162	5.69	5.28
21	9.48	7.82	136	148	5.83	5.33
28	-	-	-	-	-	-
35	9.76	8.27	151	168	5.51	5.28
42	10.94	9.35	142	140	5.49	5.25
49	9.82	8.21	158	167	5.41	5.26
56	8.8	6.18	152	181	5.46	5.23
63	7.68	6.15	161	190	5.42	5.22
70	-	6.94	152	183	5.64	5.33
77	7.6	6.0	166	185	5.56	5.25
84	7.8	6.34	162	182	5.51	5.22
Mean	9.33	7.49	152	167	5.55	5.26
Std Dev.	1.14	1.08	8.8	16.2	0.13	0.04

* Gelatine samples were dissolved and measured as daily instrument/method controls.
- Values not determined.

ADDENDUM 4. Continued.

CONTROL - SOLUTIONS OF GELATINE WITHOUT GLUCOSE INCUBATED AT 50°C.

GELATINE COLOUR = ABSORBANCE CURVE AREA 400 - 700 nm.

Sample Gelatine.	E 155 pH-6	F 155 pH-9	G 877 pH-6	H 877 pH-9
Day No.				
0	7.9	9.3	6.2	8.3
5	10.5	10.1	10.8	10.7
12	11.1	9.7	10.1	10
19	12.6	10.2	12	10.5
26	12.6	9.4	12.4	10.7
40	15.3	12.2	12.6	13.1
61	16.4	13.2	15.9	14.8

335/385 nm FLUORESCENCE INTENSITY

Sample. Gelatine.	E 155 pH-6	F 155 pH-9	G 877 pH-6	H 877 pH-9
Day No.				
0	164	144	140	187
5	181	220	206	220
12	191	230		226
19	200	266	219	232
26	217	285	240	236
40	223	309	221	262
61	233	318	252	252

1% SOLUTION pH

Sample. Gelatine.	E 155 pH-6	F 155 pH-9	G 877 pH-6	H 877 pH-9
Day No.				
0	6.31	8.81	6.33	8.8
5	6.02	8.68	6.14	8.07
12	6.1	8.59	-	7.64
19	6.14	8.66	6.14	7.78
26	6.12	8.51	6.48	7.77
40	6.07	8.50	6.18	8.08
61	6.03	*8.36	*9.09	*7.76

* Cloudy
- Value not determined.

ADDENDUM 5. Ribose gelatine interaction.

GELATINE + RIBOSE INCUBATED AT 37°C

GELATINE 155

Day	pH 6 (RA)			:	pH9 (RB)			
No	Abs-Areaϕ	Flu-Int	pH	:	Abs-Areaϕ	Flu-Int	RB-FI/100*	pH
0	9	164	6.13	:	10.9	192		9.08
1	9.6	173	6.04	:	21.8	230	4.3	8.85
2	16.7	229	5.87	:	44.2	348	8.7	8.71
3	34.5	374	5.86	:	65.1	419	13.5	8.5
4	62.6	442	5.72	:	85.4	400	16.5	8.33
5				:				
6				:				
7	177	413	5.55	:				
8	194	479	5.56	:	(122)	(425)	(23)	(7.72)
9	234.4	423	5.5	:				
10	258.3	332	5.5	:				
11	300.6	275	5.44	:				

* Fluorescence Intensity sample RB diluted 0.1 ml into 10 ml water.
ϕ Area under the absorbance curve 400 - 700 nm.
Flu-Int = Fluorescence Intensity.

GELATINE 877

Day	pH6 (RC)			:	pH9 (RD)			
No	Abs-Areaϕ	Flu-Int	pH	:	Abs-Areaϕ	Flu-Int	RD-FI/100*	pH
0	7.6	185	6.25	:	9.8	210		8.96
1	6.25	207	6.14	:	17.8	242	4.6	8.7
2	12.1	236	6.04	:	39.4	327	7.3	8.42
3	18.1	320	6.02	:	56.6	397	11.0	8.08
4	33.1	372	5.93	:	63.5	454	15.5	7.76
5				:				
6				:				
7	83.7	471	5.79	:	96.9	412	25	7.05
8	131	474	5.74	:	111	478	26	6.96
9	151.2	485	5.7	:	155.1	456	(21.4)	6.77
10	179.8	400	5.7	:	(124.6)	(417)	27.1	6.94
11	184.1	420	5.65	:				

* Fluorescence sample RD diluted 0.1 ml into 10 ml water.
ϕ Area under the absorbance curve 400 - 700 nm.
Flu-Int = Fluorescence Intensity.

ADDENDUM 5. Continued.

C O N T R O L S.

GELATINE SOLUTIONS WITHOUT RIBOSE INCUBATED AT 37°C.

GELATINE 155

Day No	pH 6 (RE) Abs-Areaϕ	Flu-Int	pH	pH9 (RF) Abs-Areaϕ	Flu-Int	pH
0	8.37	164	6.17	9.91	170	9.00
1	8.55	170	6.13	10.09	179	8.94
2	9.89	166	6.08	11.51	183	8.90
3	8.85	178	6.06	10.35	209	8.91
4	9.38	178	6.07	10.48	197	8.90
5						
6						
7	9.34	173	6.06	10.57	196	8.98
8						
9	9.43	181	6.05			
10						
11	9.76	183	6.09	11.21	204	8.97

GELATINE 877

Day No	pH6 (RG) Abs-Areaϕ	Flu-Int	pH	pH9 (RH) Abs-Areaϕ	Flu-Int	pH
0	6.90	167	6.26	9.43	197	8.94
1	7.21	199	6.15	10.14	227	8.93
2	8.45	198	6.12	10.83	230	8.90
3	8.14	206	6.13	10.48	243	8.92
4	8.08	194	6.12	10.04	230	8.88
5						
6						
7	8.53	206	6.13	10.19	244	8.93
8						
9	8.16	196	6.11	10.11	253	8.94
10						
11	8.21	192	6.13	10.19	245	8.95

ϕ Area under the absorbance curve 400 - 700 nm.
Flu-Int = Fluorescence Intensity.