A Comparative Study of Hemoglobin Estimated by the Traditional (WHO) Technique and Chemiluminesence Method (Research Note)

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ABSTRACT

The addition of hemoglobin (Hb) caused an inhibitory effect on the luminol-dependent chemiluminesence (CL) induced by the excitation of luminol by the oxidative metabolic, hydrogen peroxide, in a cell free medium. The CL was detected with an ultra-high sensitive photon counting system designed and built in the department of physiology. The inhibitory effect produced by various Hb levels was dose dependent, reproducible and linear with an r=0.997.

Hb concentration curves constructed by CL and standard Cyanmethaemoglobin (HiCN) methods were parallel.

A comparison between the inhibited CL (area under the curves), and the optical density (HiCN method) produced by same Hb levels was linear with an r=0.990.

There was no significant difference (0.1 > P < 0.5) between Hb level measured by CL and HiCN method in healthy adults samples. A point of importance, turbidity due to high leukocytes count (250 x 10^9 C/L) has no significant effect on Hb levels measured by CL and the modified HiCN methods.

These results suggest that, CL method may provide an additional reliable method for Hb estimation.

INTRODUCTION

There is a pronounced need for more sensitive, simple and rapid method for haemoglobine (Hb) determination in normal state and pathological conditions. In addition, it is well known that determination of Hb concentration by the internationally accepted standard Cyanmethaemoglobin (HiCN) (Van Kampen and Zijlstra, 1983) method may give erroneous results for blood samples from patients with high leukocyte counts, unless a special solution is used to produce the turbidity (Oesburg and Kwant, 1989). The high turbidity may result in misinterpretation, as to the diagnosis and treatment.

Depending on the well known inhibitory effect of Hb on photon count produce by luminol- dependent chemiluminesence (Gesmon, 1980; Tono-Oka, 1983). The present study introduces a method to evaluate the level of Hb from its inhibitory effect on light emission induced by excitation of luminol by the oxidative metabolic, hydrogen peroxide, in a cell-free medium. The suggested chemiluminesence (CL) technique compared with a well known WHO method (spectrophotometric, HiCN method), also the interference effect of other contents of blood (turbidity condition due to abnormal disease, i.e. at high WBCs counts) in both methods were studied.

MATERIALS AND METHODS

Apparatus Used in CL Measurement

Figure (1) represents schematically the multipurpose photon counting system which is used in this study. The system designed and built up in the Department of Physiology, College of Medicine, University of Basrah, Iraq. The system consisted of a light tight chamber made from brass, and was fitted with feed-through connectors for the circulation of water. These feed, through connectors, were made a light tight by an inclusion of a few 90° bends in the ducts. Heating water was circulated by a water thermostat and pump (Harvard Co.). An a aperture was cut in the centre of the cover brass chamber to insert Hamilton syringe into cuvette with out having to open the cover of the chamber. Cuvette is a transparent container made from a glass

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surrounded by reflectors to increase the collections of light by the photocathode of a photomultiplier tube (PMT) and fitted with a water jacket. The total volume of the cuvette is about 5 ml used to contain the sample for measurement, and was placed in a light housing impermeable to light, facing the PMT. The cuvette was located up on the light guide with 51 mm diameter made from perspex material, and this is located at the entrance to the PMT. The photomultiplier tube (PMT) type of 9635 QB manufactured by the EMI-electronic Company, (1975) was mounted inside a magnetically shielded house, which was attached by means of brass couplings to the light tight chamber, and attached to a spring in a base chamber to hold the tube firmly to light guide (Fig. 1). The PMT has the end- window type made from fused silica which passes light on to the bialkalied cathode of low back ground noise. The electrons which are emitted from a photocathode are multiplied by a thirteen stage dynodes ventian blind type with Cs Sb secondary emitting surfaces (Fig. 2). The signal output (DC current) from dynodes was amplified by a built in preamplifier, and monitored by a chart recorder (AEGER. Co.). The stabilized power, was supplied to the PMT with a constant negative high voltage (–1100 volt) (Leybold, Co).

The PMT type 9635 QB has a high cathode photoelectric quantum efficiency (Q.E) (Peak Q. E=27%). The change of Q.E with wavelength for a 9635 QB type of PMT is shown in (Fig. 3). The intensity of a maximum emission spectrum has nearly the same wavelength of a maximum response of the PMT. This results in the maximization of PMT collection of light produced by luminol-H202 reaction and gives a maximum PMT response of 27%. Figure (4) and Al-Hashimi and Mohammad, (1996) show the construction and voltage divider network of a PMT type 9635QB. The standard voltage distribution is used, based on a factor of resistance (R) equal to 100K. The current of electrons (i) collected by the anode flows to ground through a load resistance (RL) and thus, develops a voltage e = i x RL. The load resistance is often built into a preamplifier with the PMT. The current flowing in the voltage divider network is sufficient to maintain the required output current linearity at low cathode to anode voltage, particularly if zener diodes are used. At higher cathode to anode voltages, consideration must be given to power dissipation in the resistor network and generation of heat.

CHEMICALS AND REAGENTS

Luminol (Sigma Chemical Co.) in a concentration of 1.13×10^{-3} M was used. This stock solution was prepared according to the modified method of Ewetz and Thore, (1976) by dissolving 0.02 gm of luminol in 2 ml of 0.2 M NaOH and diluting to 100 ml with deionized water. The solution was stored at 4°C till used. H_2O_2 (Fluka) in 1% solution was freshly prepared prior to use. Other chemicals used were as follows: Cyanmethaemoglobin standard (57.2 mg/ 100 ml) (BDH Co.), haemoglobin standard 920 gm/100 ml) (Curtin Matueson Scientific INC), NH40H (250 ml/L), and Drabkin diluting solution.

PREPARATION OF BLOOD SAMPLES

Venous blood samples (5 ml) were obtained from healthy adults. Each sample was mixed with 5 mg (EDTA) as an anticoagulant in a measuring vial and kept at 4°C until the start of the assay. In some experiments, blood samples with high leukocytes count (250×10^9 C/L) were obtained from ten leukemic patients.

The principle of oxidation of luminol 5-amino-2, 3-dihydro- 1,4-phthalazinedione, acyclic hydrazide, by reactive oxygen species produced by the addition of H_2O_2 was employed (Gadow, 1987; Dahlgren and Sjolin, 1993; White, 1957).

The luminol- dependent CL in the luminol-H_2O_2 reaction system was measured by a multipurpose, ultrasensitive photon counting system designed and built in the Department of Physiology (Fig. 1) (Al-Hashimi and Mohammad, (in press).

The reaction mixture consisted of a medium containing 0.2 ml of luminol and 2 ml of 0.2 M NaOH placed in the reaction vessel of the system at 37°C and pH of 11. To this medium, 0.5 ml of 1% H_2O_2 was added and the resulting light was continuously recorded on a chart recorder. The recorded light (area under curve) was considered

as a relative control, which served as a standard for the inhibited light intensity produced by different Hb levels.

The results of CL were computed, when appropriate, by a computer programme, which gives the integral area under the curve of each graph.

The inhibitory effect of a series of standard Hb concentrations on the control luminol-dependent CL was measured, by the addition, each time, of 20 ul standard Hb blood to the reaction mixture. A concentration curve for the standard Hb was constructed and compared with a concentration curve for the same series of Hb levels measured by the HiCN method.

The Application of CL Method for Hb Measurement

Hb level of 21 blood samples from healthy adults was measured by the CL method, and were compared with the measurements by the HiCN method. In addition, the influence of turbidity due to high leukocytes count on Hb estimation was tested by CL, HiCN, and a modified HiCN + a drop of 250 ml/L NH_4OH) (Oeseburg and Kwant, 1989). Results were compared and anlyses for significane between different methods using student t-test.

Results were considered significant when $p < 0.05$.

RESULTS

Relationship between Hb levels and its inhibtory effect on CL:

First, we tried to detect and record a light output from luminol excited hydrogen peroxide in a cell free medium by our photon counting system (Fig. 1). A typical luminol-dependent CL as shown in (Fig. 5) was successfully recorded for the control luminol-H_2O_2 reaction system. (Depending on well known inhibitory effect of hemoglobin on a photon counts of lumonol-dependent CL (Eesmon, 1980; Tono-Oka, 1983). The inhibitory effect of a standard sample on light intensity (area under curve) was demonstrated (Fig. 5).

This inhibitory effect of a standard Hb was dose-depended (Figs. 5 and 6). The highly reproducible dose-depended inhibition of the light emitted by a series of levels of standard Hb was used to construct Hb concentration curve. The area under the CL glow curve was the most convenient parameter for quantitative measurement of the Hb% and has been used in all subsequent experiments. In (Fig. 7), the relationship was linear with a correlation coefficient of r=0.997 for Hb concentrations between 3-20 gm%. Lower than 3gm/100 ml has been tested, the limit of 0.5 gm/100 ml has been reached with good reproducibility. The coefficient of variation of the mean total light production (total area under the curve) of 21 replicate results was found equal to + 0.4%, and this decreased to +0.35% when triplicates results were averaged. The same concentration of hemoglobin content on a standard blood sample, which was measured by CL method was examined by the spectrophotometer, and according to a cyanmethaemoglobin (HiCN) method (reference method). A linear curve was obtained (Fig. not shown).

The Application of CL method for Hb measurement

Cyanmethemoglobin (a reference method) was applied for Hb estimation of the same standard blood samples measured by the CL method. The results were used to construct Hb concentration curve for comparison with the results obtained by CL method (Fig. 8). The linear curves in both methods were parallel. In (Fig. 8a), there was a linear relationship between the optical density (spectrophotometric method), and the inhibited luminescence (area under curve) produced by the same Hb concentrations. The results of the two methods were highly significant ($p < 0.001$) and show a perfect correlation coefficient of r=0.990. There was no significant difference ($0.1 < p < 0.5$) between Hb levels in all 21 blood samples measured by CL method and HiCN method (Table 1). These results indicated the reliability of CL measurements.

The increase in a concentration of hemoglobin contained in standard blood has no influence on the time showing peak, but apparently reduced the time showing shoulder as

shown previously (Fig. 6). A linear relationship was found between the Hb% and the time showing shoulder with a correlation coefficient r = 0.999 and slope = 0.14 (Fig. 9). The counts of the peak shoulder were the same for all concentrations.

The increase in a concentration of hemoglobin contained in standard blood was also followed by reduction of the area under and above the shoulder as shown in (Fig. 5A). Linear relationship was found between the area under and above the shoulder line. Also, a various Hb% of standard blood (Fig. 10) with a correlation coefficient r=0.998, slope = –2.4 (curve 2 and r=0.998, slope = –0.3 (curve 1).

Various volumes of standard blood at a same concentration (20 gm/100 ml) of a hemoglobin were found, and also have an effect on the luminol- dependent CL (Fig. 11) under the same previous conditions. The maximum peak light intensity was generally reached within 0.5 sec. and nearly remained the same for all volumes. The output CL signal declined with shoulder shape and returned to the back ground level.

The increase in the volume of standard blood apparently reduced the values of the area under the glow curve, but had no influence on the accounts peak of CL signal. A linear relationship was found between the light emission and the different volumes of standard blood (20 gm Hb/100 ml) on a logarithmic scale (1 to 100 ul), (Fig. 12). The correlation coefficient r = 0.998 and slope = –0.46 was found from this Figure.

Effect of Turbidity on Hb Estimated by CL Method

The turbidity of high leukocytes count had no significant effect ($0.1 < P < 0.5$) on Hb measured by CL method, when compared to the same levels measured by the modified HiCN method (Table 2). While a significant difference ($p < 0.001$) was observed between the same level of Hb measured by the standard HiCN method and the modified HiCN method (Table 2).

The CL method was carried out under the fixed conditions (at 37°C and pH = 11) (Tables 1 and 2). Figures (13 and 14) show the factors that influence the inhibitory effect of Hb on the luminol-dependent CL.

DISCUSSION

The present study shows the successful detection of CL and the inhibitory effect of Hb on CL by the multipurpose photon counting system built in our department (Fig. 1). CL was produced through the excitation of luminol by the oxidative metabolic, hydrogen peroxide, in a cell free medium.

From both dose-dependent and high reproducibility of the inhibitory effect of Hb on CL, an Hb-concentration curve was constructed (Fig. 7). This curve was parallel to the standard HiCN method curve (Fig. 8). In addition, we demonstrated a linear relationship between area under the curve (CL method), and optical density (HiCN method) for the same Hb levels with a high correlation coefficient of r = 0.990 (Fig. 8a). These results indicate the reliability of CL as a method for the light intensity of CL was observed in the range between 3 to 20 gm/100 ml. The study on a different triplicates measurement of Hb concentration (standard blood) shows a good linearity of CL signal against the concentration.

The minimum detectable limit for Hb is 0.5 gm/100 ml (This 0.5 gm/ 100 ml limited sensitivity for low amplifier gain, this can be increased by improving the signal to noise ratio) was observed using Cl method. High concentration of Hb reduce the intensity of light, but do not affect linearity of response. The concentration of Hb below 3 gm/100 ml produce nonlinear relationship. This nonlinearity may be due to the unaffected inhibition of the low concentration of Hb, where the light produced by the reaction of O_2 bubbles with luminol is dominate, even before mixing becomes complete, i.e. before luminol reacts with the H_2O_2. The upper limit of linear response depended on how efficiently the blood sample and H_2O_2 injection mixes with the control mixture. The linear response can be controlled by reducing the syringe drive rate or using automatic injector of fixed rate.

In conclusion, the present study suggests that the CL method is sensitive, rapid, not influenced by blood turbidity, and may provide an additional reliable method of Hb estimation. The CL method possesses many merits, as it noted experiments. First, it utilizes a small amount of blood. Second

this method is not time consuming. Third, it does not require expensive instrumentations and materials. Fourth, it is considered to be extremely accurate, sensitive and a reproducible method.

The maximum sensitivity in the spectrophotometric procedure is obtained at the wavelength where there is a greatest change in the optical density (OD) or transmittance (T%) per unit change in hemoglobin concentration. The most accurate region of the scale that corresponds to densities is between about 0.02 and 1 (60 to 10%) (Hawk et al., 1954). Reading out of this scale range represents solutions which are either too bright or dark for the most accurate measurment. For dark solutions, unit change in transmittance represents a disproportionally large change in concentration. The sample should therefore read between the scale limits specified if maximal accuracy is to be obtained (Hawk et al., 1954). The suggested CL method in sensitivity showed no limit, where the limit of scale is not restricted, and since the very low concentration of 0.5 gm/ml was detected with good reproducibility.

Table 1: Results of the Hb% measured by the CL and HiCN methods to 21 blood samples.

Blood Sample	Hb% HiCN Method	Hb% CL Method
1	12.99	12.52
2	13.60	13.22
3	16.02	15.99
4	13.78	13.48
5	12.55	13.12
6	15.66	15.60
7	13.85	13.82
8	14.72	14.81
9	12.77	12.60
10	14.61	14.56
11	12.55	12.83
12	13.63	13.30
13	14.75	14.82
14	15.33	13.16
15	12.88	12.56
16	13.71	14.01
17	16.63	16.30
18	14.36	14.39
19	12.77	13.11
20	13.56	13.50
21	15.19	15.20

Notes:

1 - The reaction mixture in CL method contained: 0.2 ml of 1.13×10^{-3} M luminol, 2 ml of 0.2 M NaOH and µl of different samples of blood 0.5 ml of 32.6×10^{-2} M of 1% H_2O_2 were injected to start the reaction.

2 - The reaction mixture in HiCN method contained: 5 ml of Drabkin solution and 20 ml of the same different blood samples which were measured by CL method.

3 - All points in both methods were done in a triplicate and reported values represent the mean.

4 - Statistical analysis using t-test between Hb g% CL method and Hb g% HiCN method: SD = 1.296, t = 14.7, $P << 0.001$, which indicated a highly significant difference.

5 - Statistical analysis using t-test between Hb g% CL method and Hb g% modified HiCN method: SD = 0.198, t = 1.569, $0.1 < p < 0.5$ which indicated no significant difference.

Table 2: Results of the Hb% to ten samples with high leukocyte count measured by the CL, HiCN and modified HiCN methods. All points were done in a triplicate, measured and reported values represent the mean.

Blood Sample	Hb% CL Method	Hb% HiCN Method	Hb% Modified HiCN Method
1	9.63	14.86	9.81
2	9.20	14.64	9.34
3	10.32	18.43	10.47
4	9.80	15.19	10.00
5	9.58	14.76	9.34
6	10.21	17.31	10.36
7	9.92	15.84	9.63
8	9.42	14.72	9.60
9	10.37	18.54	10.57
10	10.01	14.90	10.32

Notes:

The reactions mixture in a modified HiCN method contained: 5 ml of Drabkin solution treated with a drop of 250 ml/ L of ammonia solution, and 20 µl of the same different blood samples, which were measured by CL, and HiCN method.

Statistical analysis using t-test: SD = 0.266376

t = 0.8274, 0.1 < P < 0.5.

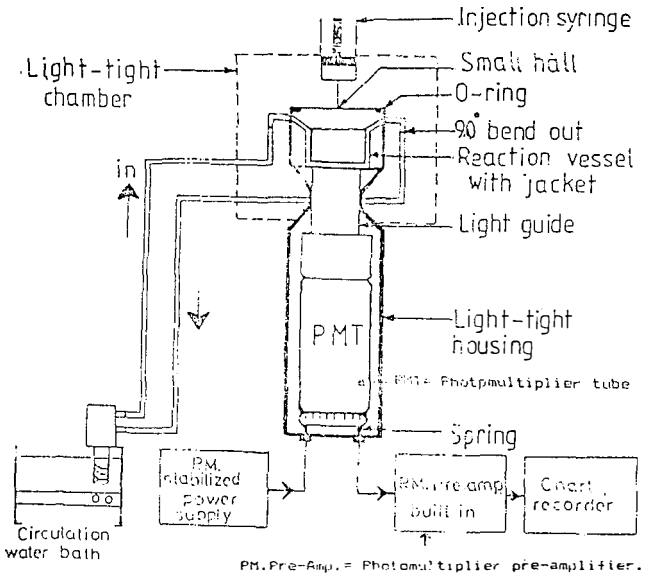


Fig. 1: Schematic Diagram of the Multipurpose Photon Counting System.

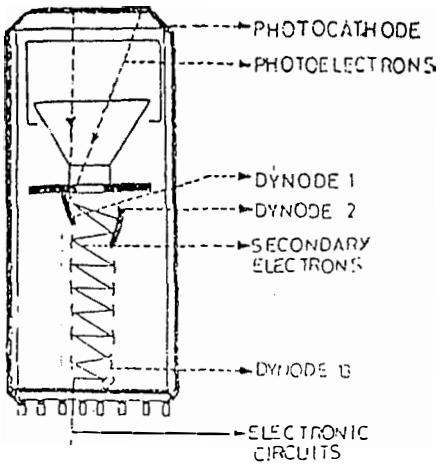


Fig. 2: Schematic Diagram of the PMT

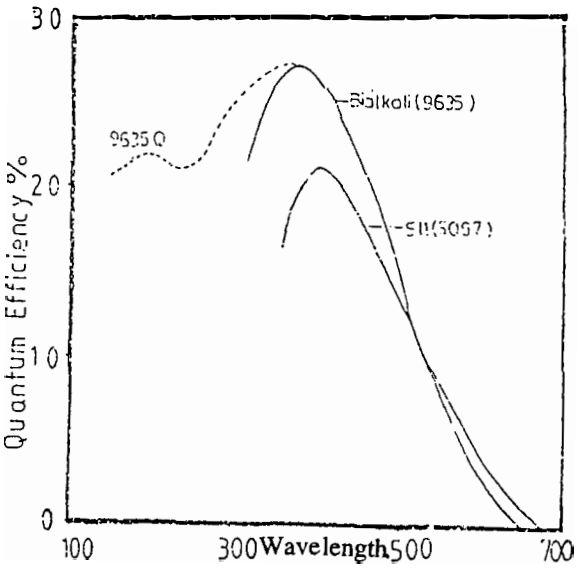


Fig. (3): Spectral response of photomultiplier tube (EMI 9635 QB), used in measurements.

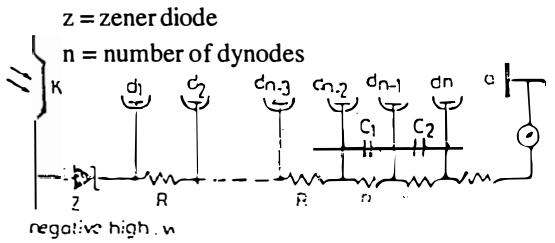


Fig. (4): Voltage Divider Set

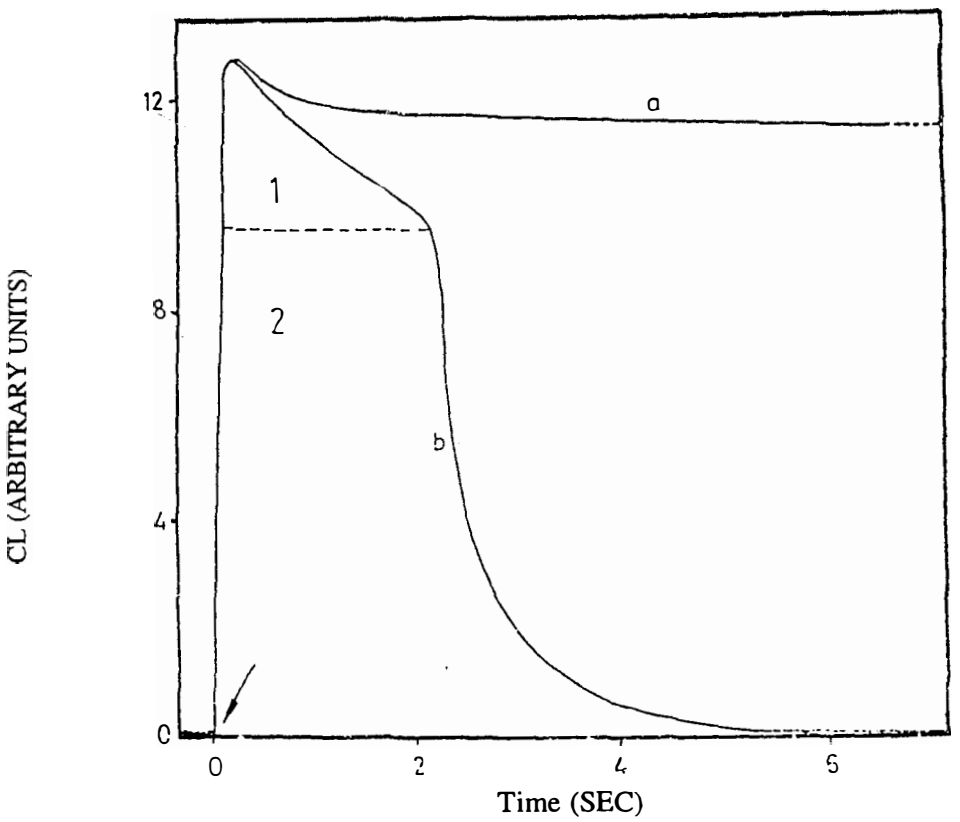


Fig. (5): The typical time course of light emission induced by luminol-NaOH-H_2O_2 reaction as a control (curve a). The reaction mixture was 2.2 ml containing 2 ml of 0.2 M NaOH and 0.2 ml of 1.13 x 10^{-3} M luminol stock solution. The arrow mark indicated that the reaction was started at zero time by injection 0.5 ml of 32.6 x 10^{-2} M of 1% H_2O_2 into a mixture. Curve b shows the typical time course of the inhibitory effect of the hemoglobin on the luminol- dependent CL with shoulder shape. The area above and under the shoulder line expressed as (1) and (2) respectively. The reaction mixture was the same as in curve (a) pluse 20 µl of a hemoglobin contained in standard blood (20 gm/ 100 ml). The reaction was started as in curve (a). The experiment was carried out at constant temperature of 37°C and pH = 11.

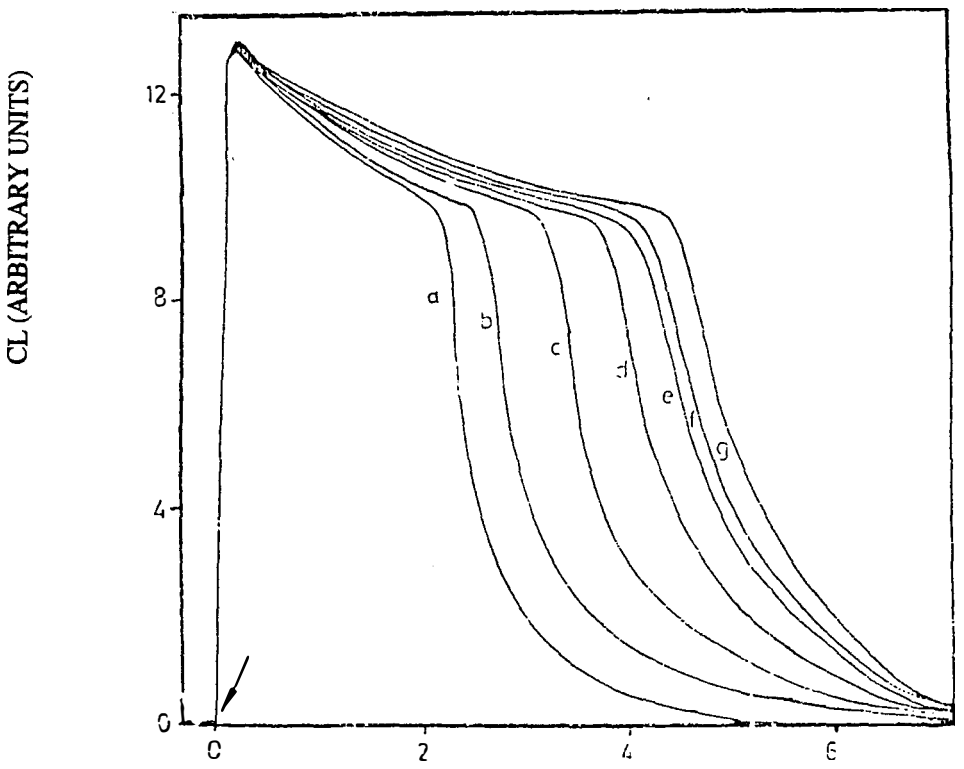


Fig. (6): Representative at the typical kinetics of the inhibitary effect of a hemoglobin (various concentration) on the CL of luminol-NaOH-H_2O_2 reaction. The reaction mixture was the same as in Fig. (5) curve (b) except various concentrations of Hb contained in standard blood samples (20 µl) were added. The reaction was started as in (Fig. 5) arrow mark). The various Hb% were 20, 17, 46, 12.66, 8.8, 5.87, 5.59 and 3 gm / 100 ml for curves a, b, c, d, e, f and g respectively. The conditions of the experiment were the same as in (Fig. 5).

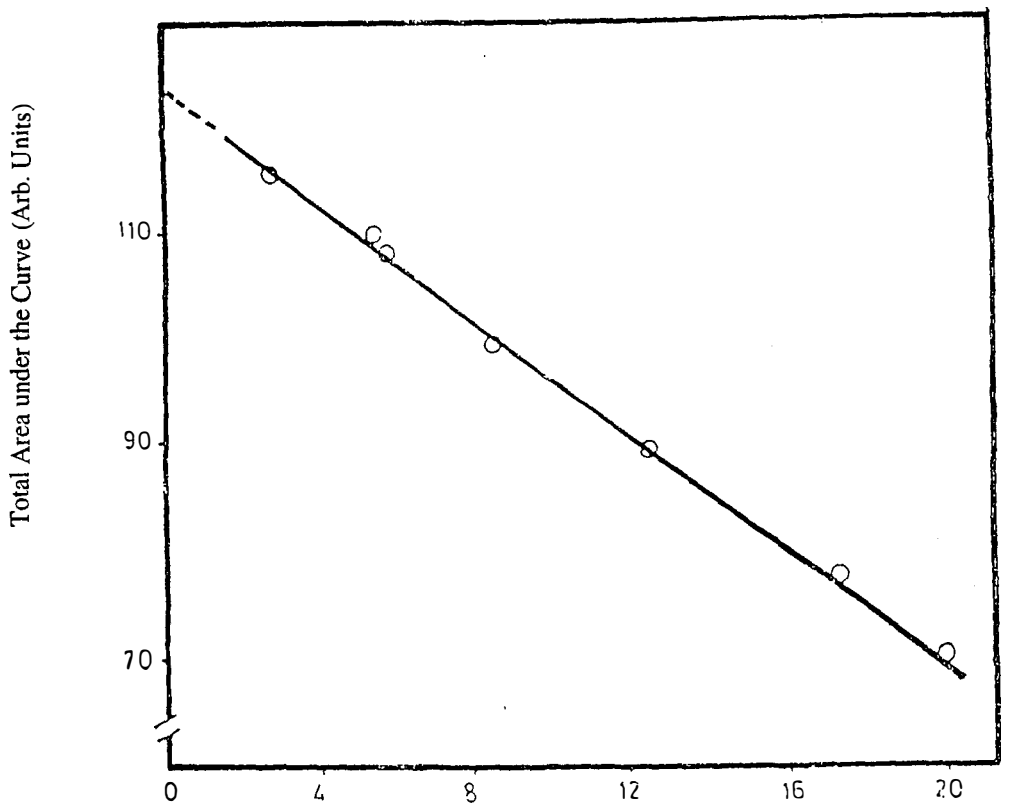


Fig. (7): The proportionality between the light emission (area under the curve in arbitrary units) and a various Hb% contained in standard blood. The regression line was plotted according to the least square method. The correlation is linear (r = 0.997, slope = –2.717). The values of ordinate were calculated by integrating the areas under the curves from (Fig. 6) in arbitrary units. All reactions were done in triplicate and reported values represent the mean ± SD, points without bars of standard deviation represent the mean value only, since SD is too small to be considered on the graph scale.

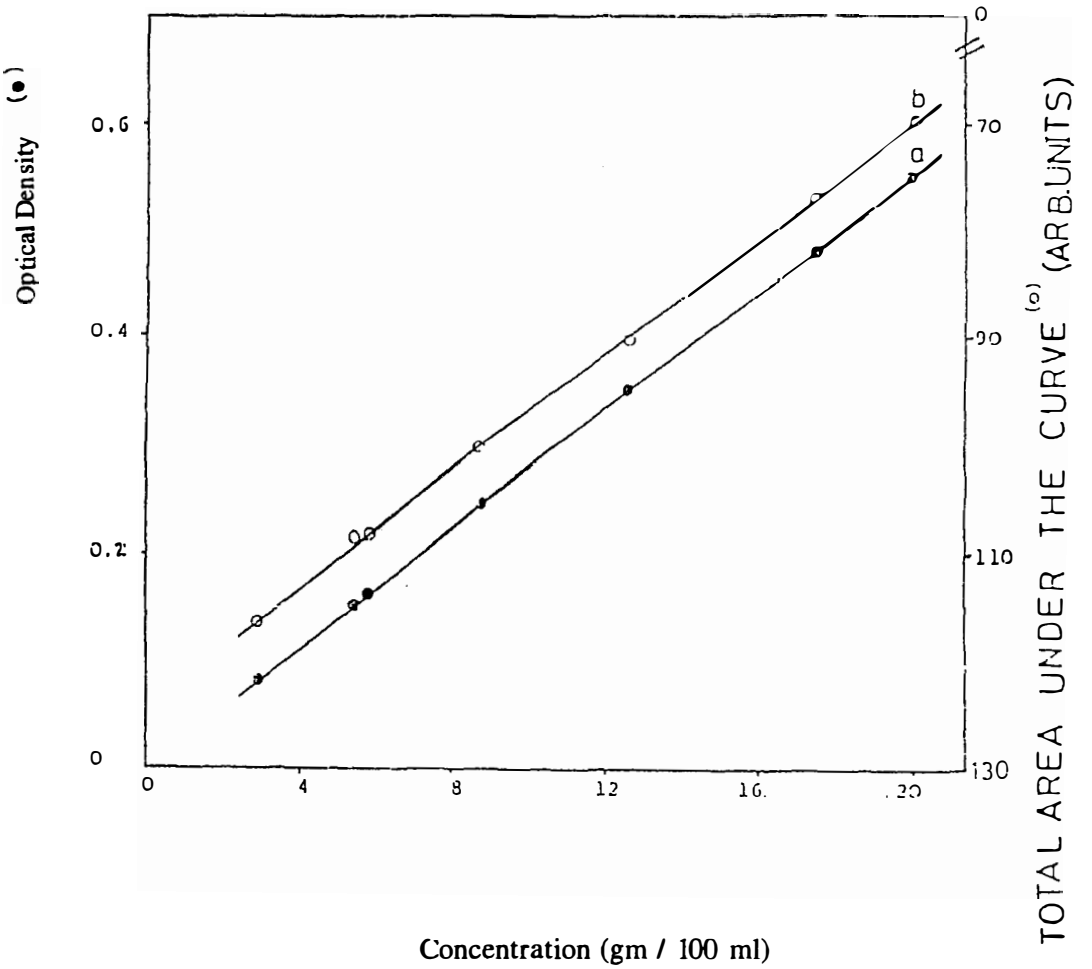


Fig. (8): Quantitative measurements of a hemoglobin contained in standard blood by CL method (o) and the reference spectrophotometric method (•). Results have been reported for the standard blood (20 gm/ 100 ml). Note that the two curves are linear and parallel.

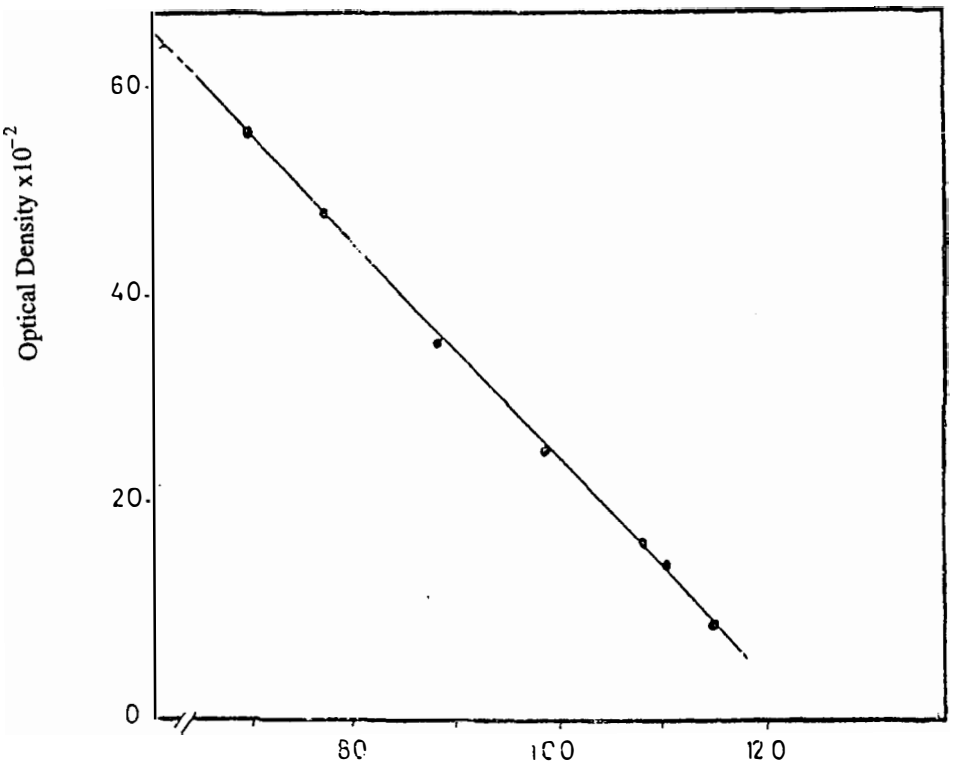


Fig. (8a): The relationship between the optical density (spectrophotometric method) and the area under the curve (CL method). The regression line was plotted according to a least square method with a correlation coefficient r = 0.997 and slope = –0.01.

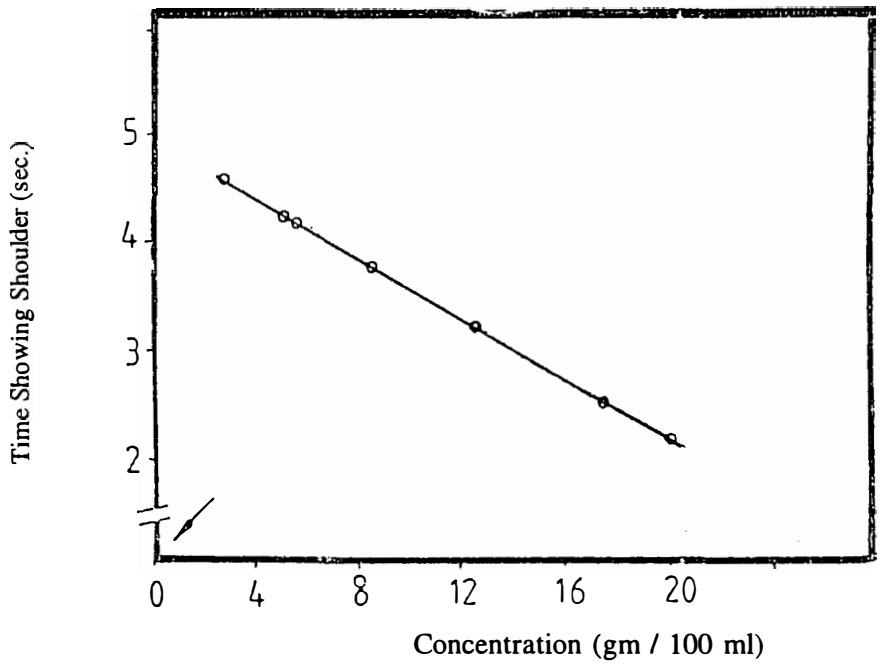


Fig. (9): The linear relationship between the Hb% of standard blood and the time showing shoulder. Each point represents the mean value of triplicate measurements. Standard deviation is too small to be represented on the graph scale. The arrow mark represents the instance of injection H_2O_2.

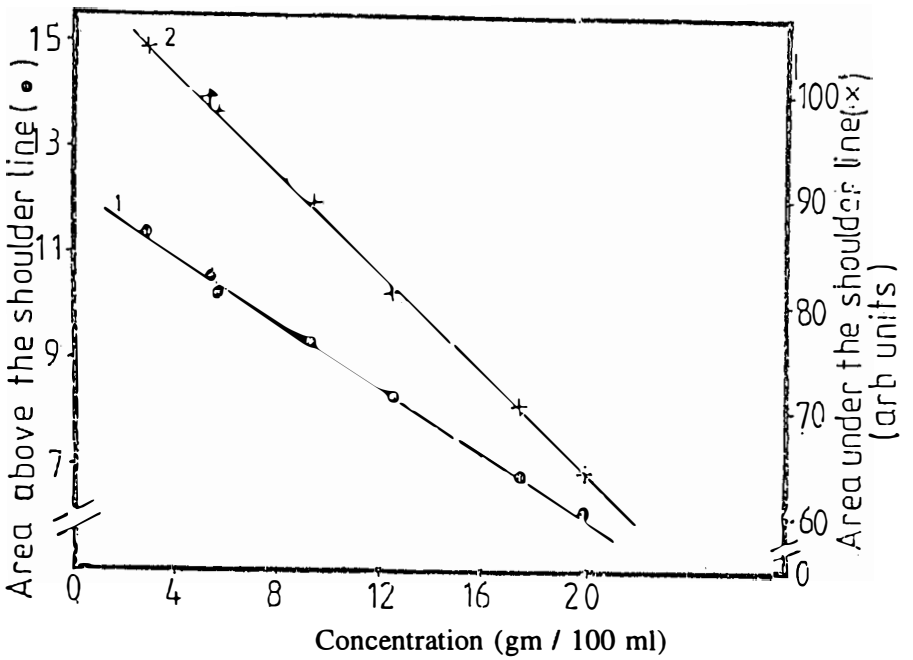


Fig. (10): The proportionality between the intensity of light emission (area above shoulder line, 1, and area under the shoulder line, 2) and the Hb% of standard blood. Both regression lines were plotted according to a least square method.

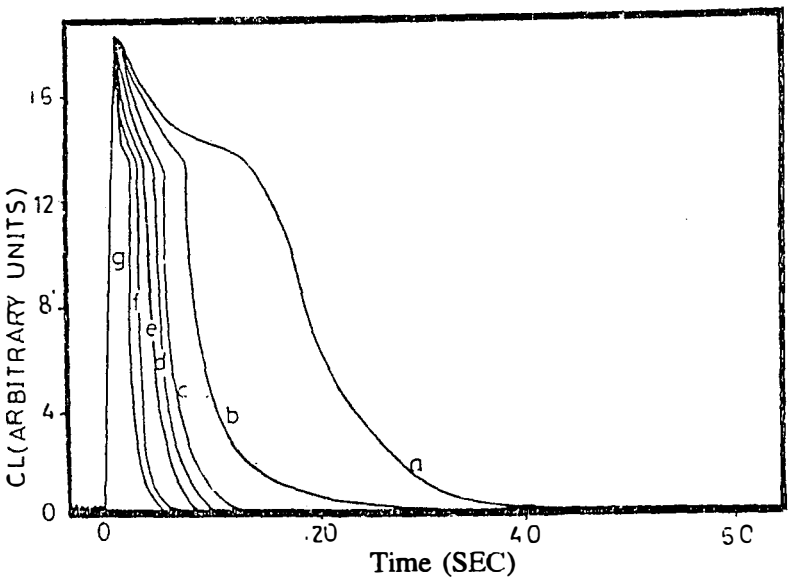


Fig. (11): The influence of the addition af various volumes of Hb contained in standard blood samples under the same conditions as previously described. The reaction medium was the same as in (Fig. 6), except that it contained various volumes of standard blood samples (20 gm / 100 ml). These were, 1, 5, 10, 30, 40, 60 and 100 µl of curves, a, b, c, d, e, f and g, respectively.

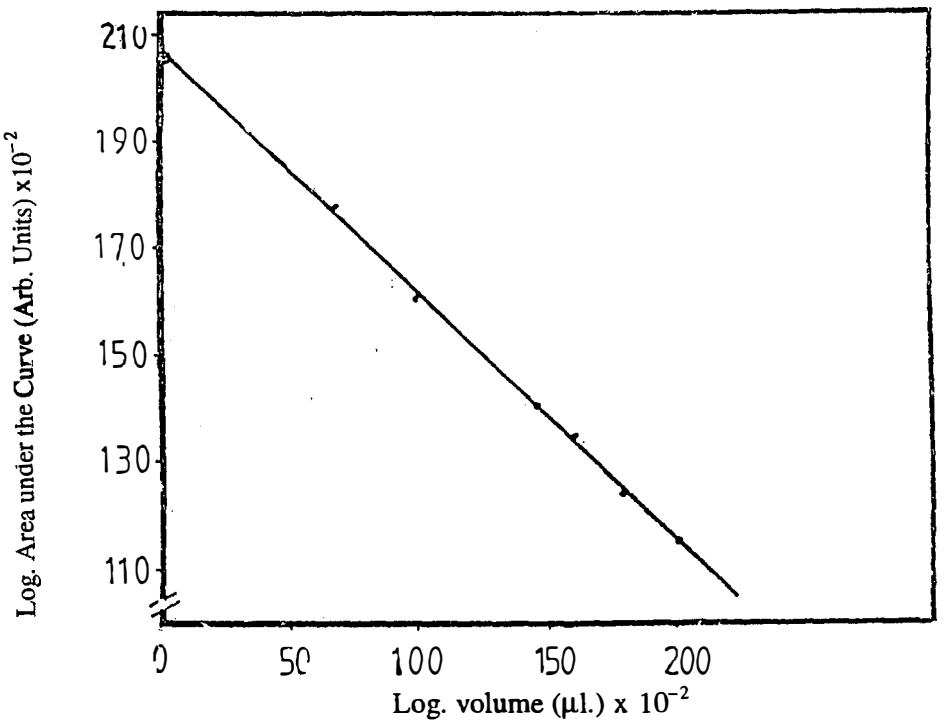
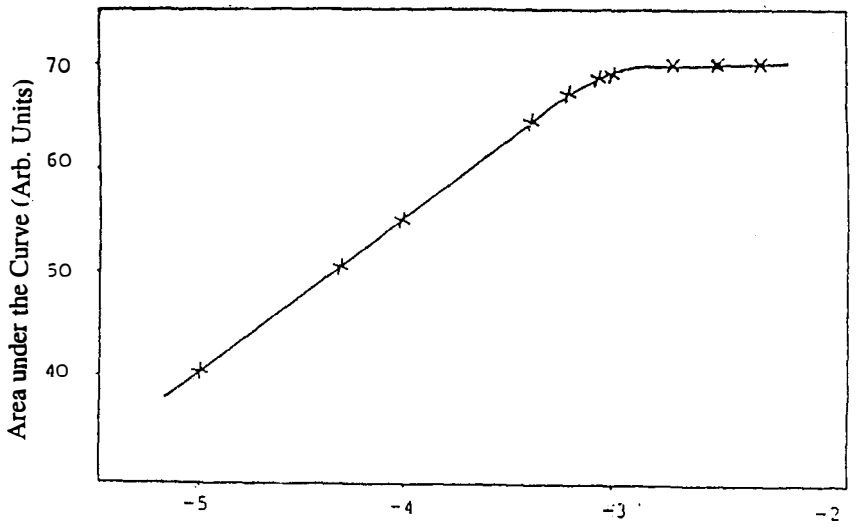


Fig. (12): The proportionality between the light emission (area under the curve in arbitrary units) and the different volumes of standard blood samples (20 gm/ 100 ml). All reactions were done in triplicate measurements and reported values represent the mean ± SD. Standard deviation is too small to be represented on the graph scale



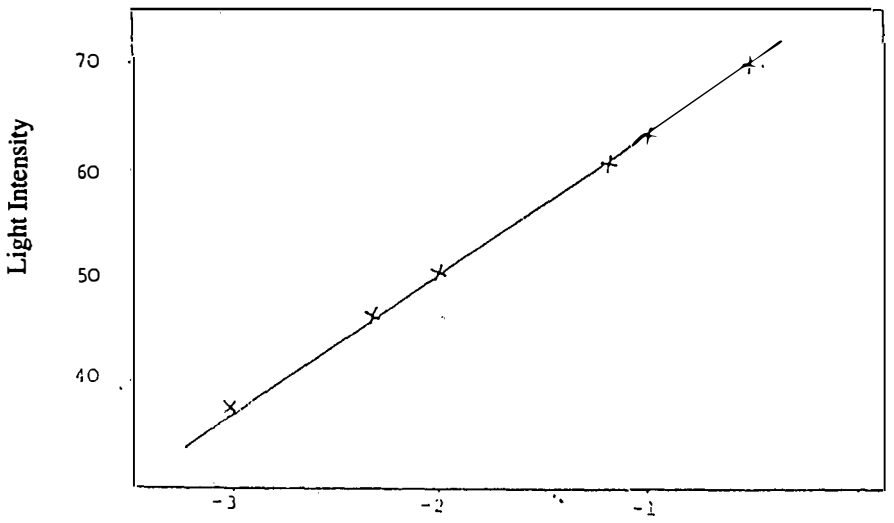


Fig. (13): The influence of luminol (upper part) and H_2O_2 (lower part) concentrations on the CL light emission in the assay of Hb% (20 gm/ 100 ml) under the same conditions used.

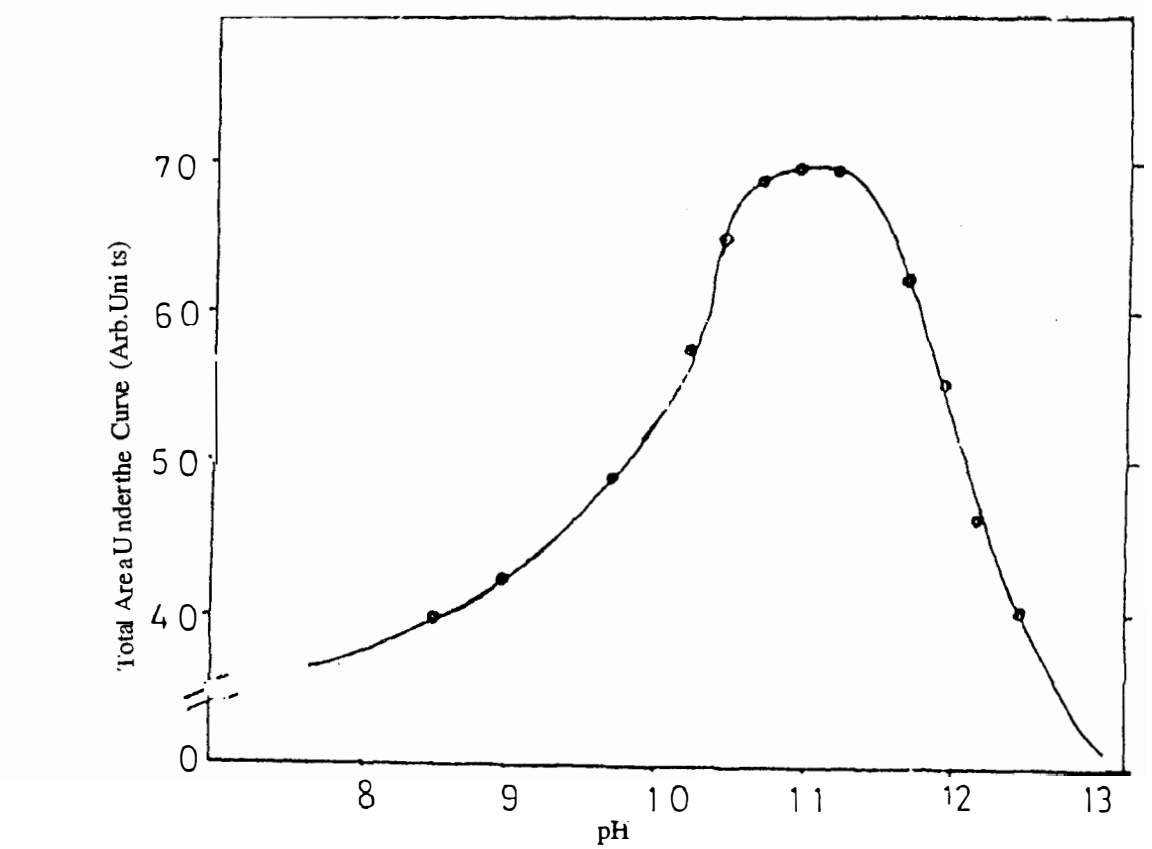


Fig. (14): Effect of pH on CL intensity from luminol - NaOH - H_2O_2 reaction with 20 µl of standard blood sample (20 gm Hb / 100 ml). The reaction medium was the same as in (Fig. 5) (curve b), except that the pH of reaction system varied. The total light production was recorded in ordinate in arbitrary units (total area under the curve) versus pH.

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تاثير إضافة خضاب الدم للمعان الكيميائي المستخلص من استثارة اللومينول بالمستقلب المؤكسد، الماء الأوكسجيني، في وسط خال خلوياً (ملحوظة علمية)

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ملخص

أحدثت إضافة خضاب الدم تأثيراً مثبطاً للمعان الكيميائي المستخلص من استثارة اللومينول بالمستقلب المؤكسد، الماء الأوكسجيني، في وسط خال خلوياً . ولقد تم قياس اللمعان الكيميائي بواسطة عداد فوتوني ذي حساسية عالية تم تصميمه وتصنيعه في قسم الفسلجة.

لقد كان التأثير المثبط لتراكيز مختلفة من خضاب الدم منوطاً بالجرعة وذا تكرارية وعلاقة خطية بمعامل ارتباط عالٍ يساوي 0.997. وكان تركيزا خضاب الدم المستخرج بطريقة اللمعان الكيميائي وطريقة السايانميثهيموكلوبين القياسية متوازيين. ولقد اظهرت مقارنة العلاقة بين تثبيط اللمعان الكيميائي (المساحة تحت المنحنى) والعتمة الضوئية (طريقة السايانميثهيموكلوبين) لنفس تراكيز خضاب الدم أنها خطية وبمعامل ارتباط عالٍ يساوي 0.990.

ولم يكن هنالك فرق احصائي مهم ($0.1 < P < 0.5$) بين قياس خضاب الدم بطريقة اللمعان الكيميائي وطريقة السايانميثهيموكلوبين لعينات دم أصحاء بالغين. ان عكرة الدم الناتجة من تعداد عالٍ غير طبيعي بحدود (250×10^9 C/L) لكريات الدم البيضاء ليس لها تأثير احصائي مهم على مستوى خضاب الدم المقاس بطريقة اللمعان الكيميائي مقارنة بطريقة السايانميثهيموكلوبين المحورة لازالة عكرة الدم. وتقترح هذه النتائج أن طريقة اللمعان الكيميائي قد توفر طريقة اضافية معتمدة لقياس خضاب الدم.

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